Clinical perspectives

Contemporary prenatal aneuploidy screening practice in Australia: Frequently asked questions in the cfDNA era

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Abstract

Cell-free DNA screening has quickly become established in Australia as an accurate – albeit costly – prenatal screening test for trisomy 21, 18, 13. It is also commonly used for the detection of sex chromosome abnormalities. The increasing number of prenatal screening pathways available to women has increased the complexity of pretest counselling. Concurrent advances in diagnostic testing with the widespread use of chromosomal microarrays create further challenges for the continuing education of clinicians and health consumers. This article aims to answer common clinical questions in this rapidly evolving field and complements the recently updated RANZCOG Statement on Prenatal Screening for Fetal Chromosome and Genetic Conditions.

Introduction

In less than a decade, the field of prenatal testing has been transformed by the introduction of cell-free DNA-based (cfDNA) screening for aneuploidy and the use of chromosomal microarrays for prenatal diagnostic analysis. The Royal Australian and New Zealand College of Obstetricians and Gynecologists (RANZCOG) statement on Prenatal Screening for Fetal Chromosome and Genetic Conditions in the Fetus has been recently updated[1]. This article

complements that statement by answering some of the frequently asked questions from College members. Further background on the biology and technical basis of cfDNA screening have been reviewed elsewhere[2]. This review assumes that population-based prenatal screening for aneuploidy is preferred as a first-line option over diagnostic testing.

How well does cfDNA screening perform in singleton pregnancies?

cfDNA screening has sensitivity of ~99% and specificity of >99% for trisomy 21 (T21) in singleton pregnancies[2], translating into a very high detection rate and very low false positive rate (0.1%). Test performance varies by chromosome, being inferior to that of T21 for other chromosomes (Table 1). As with any screening test, the chance of an affected fetus after a high probability result (positive predictive value) for T21 ranges between 46% and 90% depending on the background population prevalence[3]. False positive rates are additive for each additional chromosome so that the screen positive rate for cfDNA screening for chromosomes 21, 18, 13, X and Y is around 2% [4]. False positive results are commonly caused by a discrepancy between the placental and fetal chromosome count (e.g. confined placental mosaicism, vanished twin). cfDNA changes of maternal origin (e.g. low level maternal mosaicism or tumour) can also result in an abnormal result. Some false negative results may also have a biological cause related to discordance of fetal and placental karyotypes (eg true fetal mosaicism). Finally, laboratory errors as well as low fetal fraction should be considered as potential cause of false positive or false negative results.

How well does cfDNA screening perform in twins?

Estimates of cfDNA sensitivity and specificity are less precise for twin pregnancies than those for singletons due to smaller numbers in the available studies. A meta-analysis including over 1000 twin pregnancies calculated the sensitivity for T21 as 95.2-100%, with a low false positive rate (<0.1%) [2]. Test failures, however, appeared to be more common for twins at approximately 5%. cfDNA screening in twin pregnancies gives an overall pregnancy probability and does not determine individual probabilities for each fetus, unlike ultrasound-based screening. Diagnostic testing is therefore required on each fetus in dichorionic twin pregnancies. cfDNA screening is not recommended where there has been single fetal demise in twins, as the trophoblast from the demised twin may continue to release DNA and complicate interpretation of a high probability result.

For which conditions should cfDNA screening be offered?

Good evidence base and clinical utility

There is good evidence and clinical utility for offering cfDNA for T21, T18 and T13 for women of at least 10 weeks' gestation with a singleton pregnancy who wish to undergo screening.

Sufficient evidence base but potentially less clinical utility

Sex chromosome aneuploidy (SCA): 45X, 47XXX, 47XXY, 47XYY: Screening for sex chromosome abnormalities should be optional and pre-test counselling should include an informed decision regarding receiving information on fetal sex or suspected SCA. Due to higher maternal and fetoplacental biological variations in the X chromosome, false positive rates are higher when SCA is assessed. Biological causes of false positive results include confined placental mosaicism, maternal age-related X chromosome mosaicism, and undiagnosed maternal mosaic SCA. These lead to a lower positive predictive value for SCA, with the chances of confirmed fetal SCA after a high probability result for monosomy X, XXY and XXX reported as 26%, 84% and 50%, respectively [5]. As SCA have variable phenotypes, they have not previously been a routine component of population-based prenatal screening.

Conditions with insufficient evidence to support routine clinical application

Microdeletion syndromes: Microdeletion syndromes are the result of small, sub-chromosomal deletions that may not be detected by cytogenetic karyotyping, although would be detectable by molecular karyotyping techniques (e.g. CGH microarray). Di George Syndrome (22q11.2 deletion syndrome) is the most commonly occurring, with micro-deletions also responsible for some cases of Angelman, Prader-Willi and Cri-du-chat syndromes. The sensitivity of cfDNA screening for these conditions is lower than for common aneuploidies, and there is a paucity of clinical validation studies for microdeletion screening due to the rarity of each condition [6,7]. Furthermore, these conditions may have broad phenotypic spectrums, including asymptomatic carriers, and the true prevalence may be underestimated due to ascertainment bias away from mildly affected individuals. The addition of screening for these conditions increases the overall false positive rate with low positive predictive value.

Rare autosomal trisomies: Autosomal aneuploidies other than T21, T18, T13 represent a small proportion of all chromosomal anomalies but may be clinically relevant as they are associated with poor pregnancy outcomes including miscarriage, fetal growth restriction, fetal demise, and anomalies related to fetal mosaicism and uniparental disomy [8]. There are concerns regarding the capacity for increased false positive results due to confined placental mosaicism and undiagnosed maternal conditions.

Genome-wide subchromosomal CNVs: Detecting subchromosomal gains or losses using maternal plasma cfDNA is technically feasible. However, sensitivity will vary depending on the size of the abnormality and sequencing depth. Routine cfDNA screening for subchromosomal abnormalities is not recommended.

Why is cfDNA not diagnostic?

cfDNA has higher sensitivity and specificity compared to conventional screening tests but is not diagnostic. False positive and false negative results will occur for both biological reasons (such as confined placental mosaicism, low fetal fraction, and undiagnosed maternal conditions) and technical or statistical limitations. Overall, in Victorian women with a high probability cfDNA result, fetal aneuploidy was confirmed by invasive fetal testing in 64%, with 36% of results being false positives [9]. Again, this varies according to the condition being screened for and the background prevalence of that condition. When the diagnosis of a chromosomal abnormality will influence pregnancy management the cfDNA result should always be confirmed by a diagnostic test.

Who should be offered cfDNA screening?

Primary screening

CfDNA screening is considered suitable as a primary screening test for all women, regardless of their chance of aneuploidy. Accurate gestation and fetal number and viability should be confirmed by early ultrasound prior to cfDNA screening. Primary screening with cfDNA would result in the highest number of T21 fetuses being detected in a population, the direct cost of implementing this strategy a population level is currently viewed as prohibitive.

Secondary screening after combined first trimester screening (CFTS)

Any woman who is not sufficiently reassured by her aneuploidy probability from prior CFTS can be offered either follow-up screening with cfDNA or diagnostic testing. The trade-offs

between diagnostic testing, secondary screening with cfDNA, or no further testing will be modified by the probability from CFTS and the presence of any fetal structural abnormality. Regardless of the CFTS result, diagnostic testing with chromosome microarray analysis is the recommended management in the presence of a significant fetal structural abnormality, including nuchal translucency $\geq$3.5mm.

a) **Low probability CFTS group (T21 <1:1000)**

This group represents >85% of all screened women and the likelihood of fetal aneuploidy is very low within this group. This group need not be advised to consider cfDNA or diagnostic testing in the absence of a significant fetal structural abnormality.

b) **High probability CFTS group (T21 >1:300)**

These women should be offered either diagnostic testing or secondary screening for the common trisomies by cfDNA; noting that 1 in 72 (1.4%) will have a clinically significant chromosome abnormality not detectable by standard cfDNA[9]. The availability of cfDNA testing has seen a decline in the number of women accessing diagnostic testing after high probability CFTS but an increase in the numbers of confirmed abnormalities in those women who do proceed to diagnostic testing. This is likely due to both the introduction of nasal bone assessment into CFTS, which has reduced the CFTS false positive rate, and the use of cfDNA as a secondary screen. Approximately 20% of women who now have a diagnostic test after high probability CFTS have fetal aneuploidy confirmed [10].

c) **Very high probability CFTS group (T21 >1:100)**

This group should be more strongly advised to consider diagnostic testing. The chance of any major chromosome abnormality is this group is 18%, including a 3% risk of a chromosome abnormality not detectable on cfDNA testing [10]. These women should be given the opportunity to proceed directly to diagnostic testing to avoid undue delay in diagnosis or missed diagnosis of an atypical abnormality. Such women who have serum PAPP-A or bHCG <0.2MoM should be offered diagnostic testing as they have a 5% chance of atypical chromosome abnormality not detected on cfDNA testing (such as triploidy, trisomy 16 or mosaicism) [10].

d) **Intermediate CFTS probability group (T21 1:300 to 1:1000)**

The concept of secondary screening with cfDNA has created a new 'intermediate' CFTS category previously considered as low probability. These women can be offered cfDNA testing if they are not sufficiently reassured by their estimated CFTS probability of aneuploidy. This model balances maximising detection rates of fetal aneuploidy with the cost of cfDNA screening [11].

What is the fetal fraction and how does it relate to test failures?
Fetal fraction (FF) refers to the proportion of cfDNA derived from the trophoblast compared to total cfDNA in maternal plasma, which has both maternal and placental sources. Between 10-20 weeks of gestation, the average FF is approximately 10%. A higher FF allows better statistical distinction between euploid and aneuploid pregnancies and is considered as an important laboratory quality control measure. A low FF is the most common reason for a failed cfDNA result. The required FF threshold for cfDNA varies by assay platform but is in the range of 2-4%. The most important influences on FF are gestational age, maternal weight and fetal chromosome abnormalities. Women with a high weight should be advised that they may have a higher risk of cfDNA failure due to low FF (7% in women over 100kg and 50% over 160kg) [12].

Women with dizygotic twins may also have a lower per fetus fetal fraction and may have a higher rate of test failure. Monozygotic twins should have an adequate combined FF and theoretically comparable cfDNA test performance with singletons. IVF conceptions also appear to have a higher risk of failed cfDNA compared with spontaneous conceptions due to lower FF (5.2% vs 2.2%) [13].

Low molecular weight heparin has also been associated with an increased risk of cfDNA failure, even after controlling for maternal weight and hypertension [14]. Test failures are reported in 18% of women on LMWH, with a higher risk for those on therapeutic doses. If cfDNA is performed in such women, the blood sample should be taken just prior to the next dose [15].

How should I manage cfDNA test failures?
In a small number of patients cfDNA testing will not be successful. It is encouraged to discuss the causes of failure with the laboratory. Most commonly a low FF will be the cause. Failure rates vary by assay and range from 1.6% to 6.4% [16]. A detailed ultrasound for fetal anatomy

and aneuploidy markers should performed with diagnostic testing offered if any fetal abnormalities are detected. If ultrasound is normal, the women can be offered the choices of repeat cfDNA testing, an alternative screening method such as CFTS, or diagnostic testing. It is reasonable to include an offer of diagnostic testing after a no call result because the overall risk of aneuploidy is 2.5-fold higher among patients with failed results, due to low FF occurring in some chromosome abnormalities [17].

Should women having primary cfDNA screening have an 11-13 week ultrasound?

First trimester ultrasound has benefits in addition to aneuploidy detection, including confirmation of dates, viability, number of fetuses and detection of structural anomalies. Approximately 50% of major structural abnormalities are now detectable at 11-13 weeks, providing women with an earlier diagnosis and opportunity for diagnostic testing for chromosome abnormalities [18, 19]. Women who wish to have cfDNA as their primary screen should be offered ultrasound at 11-13 weeks, although it is recognised that this approach will increase direct patient costs [20, 21].

Should women have primary cfDNA screening before or after the 11-13 week scan?

In the early days of cfDNA screening in Australia, the average turn-around-time to receiving a result was 10 days. For this reason, early cfDNA screening at 10 weeks became the norm in order to allow for CFTS to be offered at 12-13 weeks in case of a no call cfDNA result. Current turn-around times are generally less than one week, removing some of the time-critical nature of primary screening with cfDNA. There are apparent advantages to timing cfDNA after an 11-13 week ultrasound, if early confirmation of dates and viability have been performed. In a prospective study where a detailed 11-13 week scan was performed prior to randomising to CFTS and cfDNA strategies, 2% of women were found to have a fetal anomaly at the 11-13 scan, which lead to a diagnostic rather than a screening pathway [22]. Delaying cfDNA until 12 weeks will also reduce the costs of screening aneuploid pregnancies that are destined miscarry between 10-11 weeks of gestation (6% of T21 pregnancies) [23].

Are serum markers for aneuploidy required in women having primary cfDNA screening?

cfDNA screening performs better than CFTS for aneuploidy detection and hence simultaneous screening with CFTS is not recommended as this increases the false positive rate but not the detection rate [21].

While serum markers have some value in identifying atypical chromosome abnormalities when used as part of CFTS, they do not meet accepted standards for sensitivity and specificity to justify their independent use for screening for atypical chromosome abnormalities. In fact, almost half of all atypical chromosome abnormalities (47.5%) are ascertained via ultrasound abnormality rather than serum markers or CFTS.[10]

However, storage of first trimester serum may be useful for backup CFTS screening if cfDNA testing fails. We suggest that, if interpreted as stand-alone markers for atypical chromosome abnormalities, a cut of level of 0.2 MoM is used as criteria for offering diagnostic testing as this is associated with risks of an atypical chromosome abnormality of 6.9% and 5.2% for PaPP-A and bHCG MoM respectively.[10]

The use of serum markers for conditions other that aneuploidy (such as preeclampsia) is beyond the scope of this document and is not addressed here.

Can we ignore second trimester "soft markers" in women with a low probability cfDNA result?

Ultrasound "soft markers"of aneuploidy need no longer be considered indications for invasive testing if the woman has had a low probability cfDNA result (Table 3) [20]. Some ultrasound findings such as pyelectasis, echogenic bowel, increased nuchal fold or variation in fetal biometry have independent requirements for clinical assessment and follow-up as they may indicate a fetal abnormality other than aneuploidy such as congenital infections, cystic fibrosis, fetal syndromes, skeletal dysplasia or intra-uterine growth restriction. Women with other significant structural fetal anomalies should still be offered invasive fetal testing which assesses the fetal chromosomes in more detail than cfDNA testing. In the case of bilateral ventriculomegaly (lateral cerebral ventricles ≥10mm), diagnostic testing is indicated.

Should high probability cfDNA be confirmed with CVS or amniocentesis?

Fetoplacental mosaicism is a well-known phenomenon in which the placental tissue does not reflect the true fetal karyotype, either due to confined placental mosaicism (CPM) or true fetal mosaicism. As cfDNA screening relies on DNA released from the cytotrophoblast layer of the placenta, the risk of a false positive result on cfDNA due to CPM is thought to be analagous to that observed with short term CVS culture (~1%) (Figure 1). The rate of false positive

cfDNA result due to CPM varies by chromosome and the percentage of abnormal cells [24]. The rate of mosaic CVS result after high risk cfDNA screening has been estimated to be 2% for T21, 4% for T18, 22% for T13 and 59% for monosomy X. When balancing the risks of obtaining a mosaic CVS result against delaying diagnosis until amniocentesis can be performed, ultrasound can provide useful guidance for suspected monosomy X or T13. If there is a normal appearing fetus on ultrasound there is a higher chance of CPM and, hence, an amniocentesis should be discussed as the single most informative test. For high risk cfDNA results for T21 or T18, the risk of mosaic CVS is small (2-4%) and hence offering either CVS or amniocentesis is reasonable.

What are the risks of amniocentesis and CVS?

The risk of pregnancy loss after an invasive test includes the background spontaneous miscarriage rate as well as procedure-related losses and varies according to the type of procedure, experience of the operator, gestational age, and background miscarriage risks of the population. In a recent meta-analysis including only large series, the pooled procedure-related miscarriage risks were calculated as 0.11% (1 in 909) and 0.22% (1 in 454) for amniocentesis and CVS, respectively) [25]. This represents the "best case scenario" for procedure-related losses as only data from high volume centres was included. In a 2015 survey of Australian subspecialists, the most commonly-quoted risks of miscarriage (both spontaneous and procedure-related) after a procedure were between 1 in 100-200 for both procedures [26].

Conclusion

The pace of change in prenatal testing is ever increasing as a result of advances in genomic technologies and the increasingly competitive nature of test development. The responsible integration of innovations into clinical practice will remain one of the major challenges of our era.

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Table 1: Performance of cfDNA screening

Condition	Sensitivity	False positive rate
T21	99.7% (95% CI 99.1-99-9%)	0.04% (95% CI, 0.02–0.07%)
T18	97.9% (95% CI, 94.9 – 99.1%)	0.04% (95% CI, 0.03 – 0.07%)
T13	99.0% (95% CI, 65.8 – 100%)	0.04% (95% CI, 0.02 – 0.07%)
Monosomy X	95.8% (95% CI, 70.3 – 99.5%)	0.14% (95% CI, 0.05–0.38%)

Sensitivity = true positive rate (percentage of affected fetuses correctly identified by the test);

False positive rate = number of healthy fetuses wrongly categorized as positive by the test.

(Adapted from Gil MM, Accurti V, Santacruz B, Plana M3, Nicolaides KH. Analysis of cell-free

DNA in maternal blood in screening for aneuploidies: Updated meta-analysis. Ultrasound

Obstet Gynecol 2017; 50: 302–314)

Table 2: Risk of chromosome abnormality according to combined first trimester screening (CFTS) risk group

CFTS risk	Risk of an atypical chromosome abnormality not detected by cfDNA screening (%)	Risk of any major chromosomal abnormality (%)
> 1:10	4.6	44.9
>1:50	3.1	24.4
>1:100	2.8	18.4
>1:300	1.4	8.9
>1:1000	0.6	3.2
Total CFTS population	0.1	0.4

Adapted from Lindquist A, Poulton A, Halliday J, Hui L. Prenatal diagnostic testing and atypical chromosome abnormalities following combined first-trimester screening: implications for contingent models of non-invasive prenatal testing. Ultrasound Obstet Gynecol.2018; 51: 487–492.

Table 3 Management of second-trimester isolated ultrasound findings ("soft markers" of aneuploidy) in setting of low risk cfDNA screen

Do not report (or report as normal variant)
Echogenic intracardiac focus
Choroid plexus cyst
Sandal gap toe
Clinodactyly
Evaulate as per routine clinical indications but do not consider as a soft marker for aneuploidy
Pyelectasis
Single umbilical artery
Ventriculomegaly
Echogenic bowel
Thick nuchal fold
Hypoplastic nasal bone
Shortened humerus or femur

(Adapted from Table 2 in the Society for Maternal-Fetal Medicine Consult series #42. Norton ME, Biggio JR, Kuller JA, Blackwell SC. The role of ultrasound in women who undergo cell-free DNA screening. Am J Obstet Gynecol. 2017;216(3):B2-B7.)

Figure 1.

Cell lineage in human embryos and prenatal diagnostic sampling

CVS, chorionic villus sampling; cfDNA, cell-free DNA

(See document PDF attached for the figure)

Adapted with permissions from Bianchi DW, Wilkins-Haug, Enders AC, Hay ED. Origin of Extraembryonic mesoderm in experimental animals: relevance to chorionic mosaicism in humans. Am J Hum Genet 1993; 46: 542-550.

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Undifferentiated cell

Extraembryonic ectoderm

Extraembryonic mesoderm

Embryonic ectoderm or endoderm

Embryonic mesoderm

Maternal cells
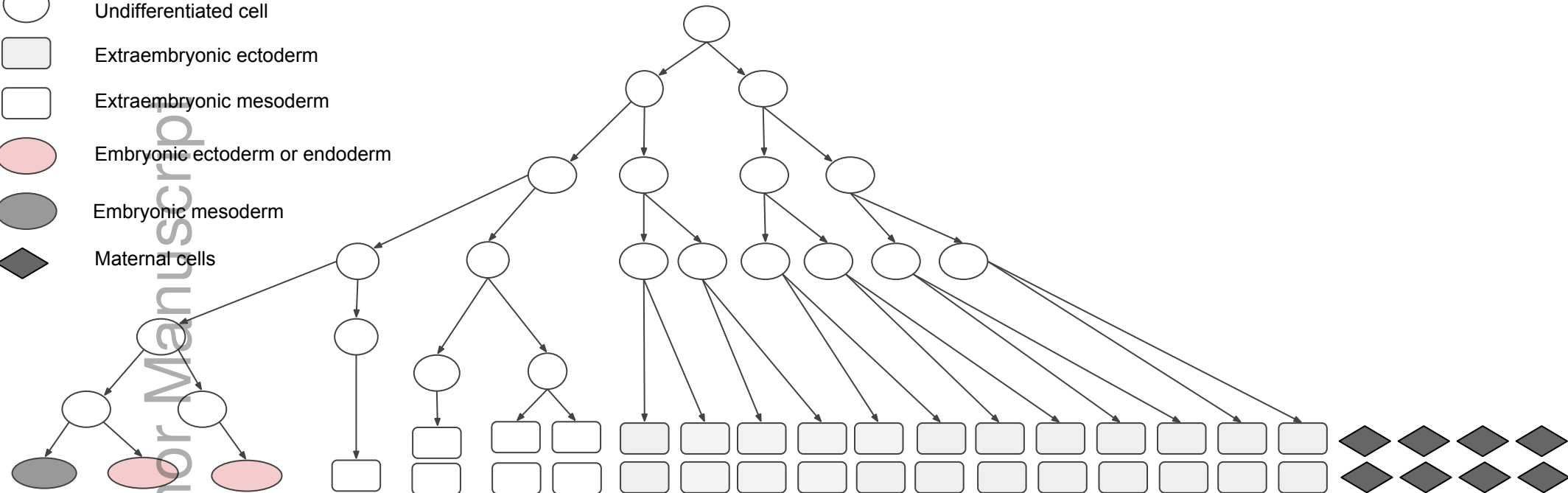
Mesoderm **Endoderm Ectoderm**

Amniotic ectoderm

Amniotic mesoderm

Chorionic mesoderm

Trophoblast (chorionic ectoderm)

Maternal cells

Fetal biopsy

Amniocentesis

CVS long term culture

CVS short term culture/ direct preparation

Maternal plasma cfDNA