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**International Society for Prenatal Diagnosis (ISPD) Position Statement:  
cell free (cf)DNA screening for Down syndrome in multiple pregnancies**

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## *Introduction/Aims*

The aim of this Position Statement from the International Society for Prenatal Diagnosis (ISPD) is to review the relevant published literature and make evidence-based recommendations regarding screening twin and triplet pregnancies for Down syndrome via cell free (cf)DNA testing. This Position Statement should not be taken to be an endorsement that cfDNA is the optimal choice for all women with multiple pregnancies. Rather, it is one of many choices that will be faced: invasive diagnostic testing for the most comprehensive and actionable information, screening tests for common aneuploidies and/or additional disorders such as microdeletion/duplication syndromes, or to choose to have no such testing. These choices, however, are best made with access to reliable and unbiased information.

Although focused on Down syndrome, trisomies 13 and 18 will also be addressed but other disorders are not, even though some cfDNA tests can identify them. The evidence will include screening performance in multifetal pregnancies for combinations of serum and ultrasound markers as well as by cfDNA testing methodologies. Current professional guidelines do not address screening multiple gestations by cfDNA testing and/or do not include the most recently published data. All compare the performance of cfDNA in twin pregnancies to that reported for cfDNA screening in singleton pregnancies. In contrast, this Position Statement will compare cfDNA testing to other screening methods available for multifetal pregnancies focusing on test characteristics such as the detection rate (sensitivity), false positive rate (1-specificity), and the test failure rate. Attention is also paid to the challenges of diagnostic procedures in multifetal pregnancies and timing of selective reduction. This review was undertaken to inform laboratories offering such testing, clinicians offering tests and receiving results, policy-makers

updating their recommendations and, most importantly, informing couples pregnant with a multiple gestation.

### *Prevalence of twin and triplet deliveries*

In 1895, Dr. Dionys Hellin published the natural rate of twin and triplet maternities as 1 in 89 and 1 in 89 x 89 (112 and 1.3 per 10,000 maternities, respectively). These estimates have been confirmed as being reasonable and can serve as a baseline rate.<sup>1,2</sup> Among dizygotic twin pregnancies, a well described positive association occurs with advancing maternal age.<sup>3</sup> In contrast, the rate of monozygotic twinning is relatively constant at 35 per 10,000, regardless of race, geography or maternal age.<sup>3</sup> The natural proportions of twins are about one-third monozygotic and two-thirds dizygotic. The lowest rates of multifetal deliveries occur in Asia with 30-40 per 10,000. Much higher rates of 180 per 10,000 are reported in central Africa.<sup>4</sup> Some suggest the rate of Down syndrome in twin term deliveries (one or both affected) may be higher than in singleton pregnancies, after accounting for zygosity.<sup>5,6</sup> However, observational data suggest the ratio may actually be close to one.<sup>7</sup> Modeling in this current report assumes the rates for the common trisomies to be the same in twin (one or both affected) and singleton deliveries for a given maternal age.

Figure 1 shows the change in twin and triplet liveborn delivery rates in Australia, the Netherlands and the United States from 1980 to 2018. These mirror the increasingly higher rates of multiple gestations around the world<sup>8-11</sup> and are likely due to several factors. Both Black/African Americans and Hispanic mothers are more likely to have spontaneous multiple gestations and these groups may now represent a greater proportion of the population. Older women are also more likely to have spontaneous multiple pregnancies and, in many countries, the average maternal age at delivery is rising. Maternal age may account for about one-third of the increase seen in the US.<sup>12</sup> The factor responsible for most of the remaining increase was

the introduction of *in vitro* fertilization (IVF) in the 1980s.<sup>13</sup> Perhaps half of these services are provided to women age 35 and older. The more recent decline in twin and triplet pregnancies may reflect a temporal change in IVF practices relating to single embryo transfer guidelines.<sup>14</sup>

The conception rate for twins is greater than the birth rate as the disappearance of gestational sacs or embryos after documented heart activity is not unusual. This is known as co-twin demise and occurs in between 30% and 40% of twin sacs or embryos.<sup>15</sup> The precise mechanisms and pathophysiology are obscure but the remaining placenta may still be functional for some time. When death occurs beyond the first trimester,<sup>16</sup> the survivor has a higher risk for low birth weight and small for gestational age.<sup>17-19</sup> The demised or vanished twin can explain discrepancies between cfDNA test results and the fetal karyotype.<sup>20,21</sup>

#### *Changes in IVF practices over time*

Beginning in the 1980s, IVF involving multiple embryo transfers was routine due to low implantation rates.<sup>14,22</sup> In the last decade, a decrease in the rates of twins and higher-order multiple deliveries occurred, although still remains higher than would naturally occur (Figure 1). The 2016 guideline from the American Society of Reproductive Medicine and the Society of Assisted Reproductive Technology recommends decreasing the number of embryos transferred per cycle.<sup>14</sup> Other factors also decreasing the rate of multiple pregnancies include promotion of single embryo transfers due to the technological advances of culturing embryos to the blastocyst stage and ensuring higher per embryo implantation success. Estimates suggest that more multiple gestations now stem from ovulation induction/intrauterine insemination cycles

than from IVF.<sup>23</sup> Lastly, another contribution to the decrease is the increase in multifetal pregnancy reductions being performed.<sup>24</sup>

Twin pregnancies conceived with IVF are more than 95% dizygotic, higher than the two-thirds expected in those naturally conceived.<sup>25</sup> Dizygotic twins are also more common in pregnancies conceived with ovulation inducing agents alone (without IVF) since these drugs increase the likelihood of ovulation and fertilization of multiple oocytes.



### *Invasive procedures and diagnostic testing in multiple pregnancies*

Prenatal diagnostic testing remains the gold standard for obtaining genetic information about the pregnancy. Either chorionic villus sampling (CVS) or amniocentesis can be offered but there are special considerations compared to singleton pregnancy. Whether the pregnancy is monochorionic or dichorionic is optimally determined by ultrasound at 9 to 12 completed weeks.<sup>26</sup> When a patient with a multifetal pregnancy chooses a diagnostic procedure, each fetus should be identified and its placental position documented. This assures each fetus is sampled only once and that the results can be accurately attributed to the correct fetus following prenatal diagnosis one to two weeks later. Ideally, the specialist who performed the ultrasound and mapping should perform the diagnostic procedure.

Monochorionicity indicates monozygosity and this implies that the two fetuses will have identical genomes. Under this assumption, a single transabdominal needle entry or single transcervical catheter entry can be performed, decreasing the risk of pregnancy loss. However, monochorionic pregnancies with discordant fetal anomalies due to post-zygotic events such as non-disjunction and twinning errors do occur.<sup>27-32</sup> For this reason, amniocentesis may be the more appropriate procedure to assure each fetus is sampled independently. A maternal fetal medicine or other specialist provider who performs diagnostic procedures frequently in multiple pregnancies can minimize the chance of a sampling error.

About 90% of dichorionic twin pregnancies are dizygotic with the remainder monozygotic. For dichorionic pregnancies, both placentas should be sampled, typically between 11 and 13 completed weeks. CVS via transabdominal or transcervical approaches can be used, including a combination of both. The risk of CVS cross-contamination (sampling the same fetus twice) is

approximately 1%.<sup>33</sup> If there is a karyotype abnormality in one fetus, this allows time for a selective reduction.

In triplet pregnancies it is important to perform an 11 to 13 week ultrasound to determine chorionicity and map out the location of the fetuses to their respective placentas to allow for successful diagnostic testing. Because of the technical expertise needed to perform CVS in triplet pregnancies and the possibility of cross contamination,<sup>34</sup> a perinatologist or other specialist experienced in such procedures is preferred. Data on zygosity in triplets conceived spontaneously versus via ART remain limited. Among ART triplets, MZ twin pairs were markedly less likely than among spontaneous triplets (6.5% vs 48%, respectively).<sup>35</sup>

#### *Unintended loss after selective termination in multiple pregnancies*

Selective termination is defined as the termination of an anomalous fetus in a multifetal gestation. These procedures are generally performed in the late first or early second trimester after one (or more) fetuses have been diagnosed. There are various procedures that can be used depending on the provider's preference, the gestational age, and zygosity. A recent report<sup>36</sup> of one tertiary referral center's experience with dichorionic diamniotic twin reductions to singleton reductions compares outcomes between procedures performed in the late first trimester (12-14 weeks) with those performed in the early second trimester (16-20 weeks). The earlier reductions (N=172) were often due to patient's request or abnormal ultrasound findings while the latter group (N=76) were mainly due to structural or genetic abnormalities. Fetal loss before 24 weeks was similar (0.6% early and 1.3% late,  $p=0.52$ ) but with later reductions, neonatal morbidity (2.9% vs 10.7%,  $p=0.025$ ) and delivery prior to 35 weeks gestation (1.8% vs

12%,  $p=0.002$ ) were higher and birthweight was lower (2,800 vs 3,025 g,  $p=0.012$ ). There are limited data regarding selective termination in monozygotic twins. One study<sup>37</sup> did provide results of selective termination in complicated monochorionic twin (N=73) and triplet (N=7) pregnancies undergoing cord coagulations at two tertiary fetal medicine centers. The gestational ages at procedure ranged from 15 to 29 weeks, likely later than the gestational age range for a similar procedure after diagnosing a common aneuploidy via cfDNA screening. Loss rates of twin and triplet pregnancies were 16% and 21%, respectively. Overall, 79% of deliveries occurred after 32 weeks. One year follow-up found developmental delays in 8%, mainly in the group delivering prior to 29 weeks.

#### *Comparative performance of age, biochemistry and ultrasound screening tests for Down syndrome in multiple pregnancies*

The use of only maternal age to screen for Down syndrome (or other common trisomies) is easy and inexpensive but has relatively poor performance. For example, using age 37 or older at delivery in the 2018 US population, 59% of affected twin and 66% of affected triplet pregnancies would be identified with false positive rates of 15% and 19%, respectively. As with singleton pregnancies, approaches to modifying the maternal age alone risk for Down syndrome also exist for multifetal pregnancies.

One summary of published studies reviewed serum screening occurring in the first, second, or both trimesters in twin pregnancies (Figure 2).<sup>38</sup> With second trimester analytes, a trade-off occurs: a similar or higher detection rate, but at lower false positive rates of 5% to 12% compared with age alone. First trimester incorporation of nuchal translucency (NT) with

maternal age increases detection to 93%, but with a 10% rate of offering diagnostic testing. NT alone offers 80% detection at a reasonable 5% false positive rate. Notably, adding the first trimester serum markers does not improve screening appreciably in multifetal pregnancies as they are pregnancy-specific rather than fetal-specific. The integrated test is the most complex testing methodology but does allow for up to a 93% detection rate at a 5% diagnostic testing rate in twin pregnancies and many of those affected fetuses can be detected in the first trimester. The reported performance of cfDNA screening for the common trisomies in twins is also included (Figure 2, upper left) but is discussed in a later section.

Less information is available for screening twin pregnancies for trisomies 18 and 13, but both can be best identified using first trimester NT measurements, biochemical measurements and maternal age.<sup>39,40</sup>

#### *Professional Recommendations for cfDNA screening in multiple pregnancies*

The use of cfDNA screening for Down syndrome in multifetal pregnancies has received increasing attention in recent years. Table 1 provides a brief summary of published recommendations from ten professional societies.<sup>38,41-49</sup> The search was limited to those published in 2015 or later. The majority of recommendations published prior to 2018 tended to not address this issue. Many (4 of 10) recommend further investigation (2016-2018).<sup>38,42,44,48</sup> Others were silent on the issue (3 of 10)<sup>43,45,47</sup> or were opposed to testing in twins (1 of 10).<sup>46</sup> One other recommends screening for Down syndrome in twins but not for trisomies 18 or 13 due to lack of data.<sup>41</sup> The earliest recommendation, published in 2015,<sup>49</sup> implied screening was acceptable for all three disorders by stating performance in twins was similar to that in singleton pregnancies. Only two recommendations directly addressed triplet pregnancies. Both

recommended the use of first trimester ultrasound markers at 11 to 13 weeks gestation and did not address cfDNA testing.<sup>42,49</sup> Since this document was drafted, the pre-publication of a new ACOG Practice Bulletin was released.<sup>50</sup> That document makes a level B recommendation that “cell-free DNA screening can be performed in twin pregnancies”. In addition, England recently released a plan to employ cfDNA testing in both singleton and twin pregnancies.<sup>51</sup>

#### *Methodologies for cfDNA screening among twin pregnancies*

Maternal plasma cfDNA screening tests measure the distribution of nonmembrane bound DNA fragments derived from the various human chromosomes. A fetal chromosomal aneuploidy is suspected when the amount (percentage) of cfDNA fragments from a particular chromosome differs from the amount expected for a euploid karyotype. The test rationale behind fetal trisomy detection is based on the assumption that the mother is euploid. Hence, any deviations in the chromosomal DNA amounts are first assumed to be of fetal origin and thus warrant further definitive diagnostic testing.

Several cfDNA test methodologies are available for aneuploidy screening and they are generally categorized as whole-genome or targeted approaches.<sup>52</sup> Whole-genome methods are based on massively parallel sequencing of large numbers of randomly captured cfDNA fragments to identify and then count the cfDNA contributions from each human chromosome.<sup>53</sup> Targeted approaches selectively analyze cfDNA from the chromosomes of interest (e.g., chromosomes 21, 18, 13, X and Y). The cfDNA fragments from those chromosomes are first amplified and then identified by sequencing or microarray.<sup>54</sup> After whole-genome or targeted DNA data are generated, statistical analyses are performed to determine if the amount of DNA

from one chromosome is relatively increased or decreased compared with the expected amounts based on chromosome size. In one variation of the targeted approach, polymorphic DNA regions on the chromosomes of interest are selectively analyzed. Ratios between heterozygous alleles are determined for many single nucleotide polymorphism (SNP) sites. Aneuploidy is suspected when the allelic ratios of one chromosome deviates from the values of the other chromosomes.<sup>55</sup> When using this approach, caution is needed for situations where the SNP inheritance pattern between the fetus and pregnant woman may be confounded, such as in pregnancies involving egg donation, surrogacy, or consanguineous parents.<sup>55</sup>

Published summaries<sup>56,57</sup> include testing by whole-genome as well as targeted approaches. The combined detection and false positive rates for trisomies 21, 18 and 13 among twin pregnancies appear comparable between methodologies. There are, however, insufficient data to compare the performance between monozygous and dizygous twins and the challenge is in those dizygous twins where each fetus is genetically different. Optimally, test protocols should ensure that the status of each fetus is actually assessed<sup>55,58</sup> and to achieve this goal, adequacy of fetal fraction would be an important factor.

### *Cell free (cf)DNA screening performance in twin pregnancies*

A formal review of the literature regarding the performance of cfDNA testing in twin pregnancies was published in 2019.<sup>56</sup> That review included only population-based studies and did not include eight additional studies that were either of case/control design or were published after that review was completed.<sup>53,55,58-63</sup> Table 2 is a compilation of results from both the formal analysis and the eight additional studies. For example, results from the formal analysis (with minor modifications) shows a total of 3,780 twin pregnancies with 56 having at least one fetus with Down syndrome. Among the 56, there was one false negative; two false positive results also occurred. The results for the eight additional studies include 40 additional affected twin pregnancies with 28 having a Down syndrome fetus.

The results from the two groups of publications were similar, with a total of 4,815 twin pregnancies tested with results. Together, 20% were monochorionic and 8% (9/117) of affected twin pregnancies involved an aneuploidy in both fetuses (sTable 1). A total of 117 twin pregnancies had at least one fetus with a common autosomal trisomy (84 Down syndrome, 29 trisomy 18 and 4 trisomy 13) with detection rates of 98.8%, 93.1% and 75%, respectively. The overall false positive rate among unaffected pregnancies was 0.29%. These chromosome specific detection and false positive rates are also shown in the upper left hand corner of Figure 2. The estimated first trimester prevalence for these three disorders in twins are estimated to be 1:340, 1:1,100 and 1:3,500 (same as for singleton pregnancies) and the false positive rates are set to 0.1% for each chromosome. The corresponding positive predictive values for successful cfDNA testing in twins are approximately 75%, 47% and 19%, respectively. The lower predictive value for trisomies 18 and 13 are mainly due to their lower prevalences. These

predictive values are expected to be similar to those found in screen positive singleton pregnancies.

One publication<sup>64</sup> was identified as this review was being completed and was not included in Table 2. This US group matched cfDNA testing results with karyotyping results performed in the same laboratory. In such a design, identifying all false negative results would be difficult. Overall, detection rates for trisomy 21, 18 and 13 were high at 98% (48/49), 100% (22/22) and 100% (6/6), respectively; consistent with the findings in Table 2. However, the overall false positive rate of 6.8% (20/294) is considerably higher than those in Table 2.

#### *Fetal fraction measurements among multiple pregnancies*

The portion of cfDNA in the maternal sample contributed by the fetal compartment is called the fetal fraction. In singletons, a low fetal fraction is associated with test failures or false negative results for trisomies 18 and 13, but less so for Down syndrome. Most cfDNA screening tests estimate the fetal fraction and set a minimum threshold as a quality control measure. When specified, minimum requirements for singleton pregnancies range from 2.8% to 5%.<sup>53-55</sup> These values would also apply to monozygous twins. For dizygous twins, it would be preferable to measure fetus-specific fractions or set the minimum requirement higher. The fetal fractions between each fetus of a dizygous pair are highly correlated ( $r=0.86$ ), but still differ by 1.5-fold or more in 10% of cases.<sup>55,58</sup> The average fetal fraction for each twin is lower than that for singletons (Table 3), because the total fetal fraction for twins is only 1.4 to 1.6 times higher. The presence of trisomy 13 or 18,<sup>65</sup> which tend to have even lower fetal fractions, further confounds interpretation.



In multifetal pregnancies, there is a wide range of methodologies used for fetal fraction determination and reported values may reflect the total pregnancy or fetal-specific fraction (Table 3). Quantifying chromosome Y cfDNA is common<sup>61,66,67</sup> and when both twin fetuses are males, the result is the total fetal fraction. When only one fetus is male, the result is specific to that fetus only. Analysis of placental methylation DNA signatures,<sup>60</sup> quantification of shorter segment DNA,<sup>63</sup> and cfDNA sequence read distributions on select autosomal regions<sup>57</sup> all can provide the total fetal fraction regardless of fetal sex and number of fetuses. Lastly, the ratio between non-maternal to maternal SNP alleles provides estimates of the fetal fraction.<sup>68</sup> Each fetus in a dizygous twin-pair will have different SNP profiles while they will be identical for monozygous twins (total fetal fraction). For dizygous twins, SNP allelic determination can provide a fetal fraction for each fetus and can also determine zygosity.<sup>55,58</sup>

Any one or more of the fetal fraction measurement methods have been used in combination with whole-genome or targeted cfDNA aneuploidy screening tests (Table 3).<sup>69</sup> While some programs use a fixed fetal fraction cut-off to determine adequacy, other methods are also utilized. In multifetal pregnancies, this cut-off is dependent on whether total fetal fraction or individual fetal fraction is measured and whether the twins are likely monozygous or likely dizygous. For monozygous twins and individual fetal fraction measurement of dizygous twins, the same fetal fraction cut-offs as for singleton pregnancies are often adopted. Test protocols that measure total fetal fraction in twins often set thresholds twice that of singleton pregnancies.<sup>60,63,70</sup>

#### *Failure rates for cfDNA testing in multiple pregnancies*

Testing cfDNA may, at times, fail to produce a clinically useful result. These results are referred to as “no call” or “test failure” rates<sup>54</sup> and are often due to insufficient fetal fraction or other sample quality issues, such as inadequate blood volumes or other technical issues. After a test failure, a duplicate sample could be tested or a second blood drawn. The repeat test will resolve a portion of such cases. The initial no call rate for cfDNA aneuploidy screening among twin pregnancies ranges from 1.6% to 13.2%, with a median of 3.6% (Table 3). Insufficient fetal fraction is the main reason.<sup>54,55,57,61,66,69,71,72</sup> Five studies<sup>54,57,66,71,72</sup> provided revised failure rates in 2,938 twin pregnancies when a second blood draw was offered to 197 of them (Table 3). Between 83% and 100% of those women provided a second sample (overall 91%, 179/197). Success rates in the repeat samples ranged from 50% to 83% with a median of 57% (overall 58%, 103/179). The median failure rate was reduced from an initial 5.6% to a revised 3.1% (45% reduction). Two studies<sup>71,72</sup> included direct comparisons with singleton pregnancies and the rates in twins were 3.3 and 3.2 times higher than in singletons. Multivariate analyses identified high maternal weight and IVF as important predictors of test failure rates.<sup>54,57,71,72</sup> If the pregnancy is still within the 11 to 13 week window, obtaining an NT measurement (with or without biochemistry) may be helpful.

#### *Screening performance in triplet pregnancies*

Triplet pregnancies are uncommon. Among 10,000 deliveries, about 150 twin and 3 triplet pregnancies would occur. Assuming the first trimester prevalence of Down syndrome in triplet pregnancies was 1:340 (same as for singletons), then 10 cases would occur among 3,400 triplet pregnancies. To obtain that number of cases would require a total screened population of about

11 million. However, even this might not be sufficient as current professional society guidelines either recommend against, or are silent on, cfDNA testing in this population even though larger numbers of cases were available in the literature (Table 1). Alternatively, perhaps a majority of triplet pregnancies are now due to IVF and some may have had pre-implantation genetic testing for aneuploidy, resulting in a lower than expected rate of trisomies. Two studies reported cfDNA test results in more than 10 triplet pregnancies,<sup>63,70</sup> but none were reported to be screen positive or from a known trisomic pregnancy. Thus, observed detection rates for Down syndrome and other common trisomies are absent. Test failure rates are likely to be higher than for twins (16.5% and 21.3%, Table 3), especially when fetus-specific fetal fraction estimates are not available. However, there are data showing that cfDNA testing can correctly identify the fetal sex in all triplet combinations (83 of 85 calls).<sup>70</sup>

Based on our current knowledge of how cfDNA testing distinguishes the underlying genetics between mother and fetus, one could extrapolate this knowledge from screening singletons and twins to a theoretical performance in triplet pregnancies. Assume each fetus in a triplet pregnancy contributes sufficient cfDNA to result in a sufficient fetus-specific fetal fraction (e.g., at least 4% per fetus). Then this can be viewed as the unaffected fetus (or fetuses) and the mother all having two copies of chromosome 21, while the affected fetus has an additional copy. At least for shotgun methods, the screening performance should approach that found for twin pregnancies.

*Patient education materials for cfDNA screening in twins*

Educational and resource materials were identified from five commercial laboratories and two academic sites through online searches.<sup>73-79</sup> In some instances relevant materials were readily identified via a built-in web site search utility. When this was not present, the site was searched manually, a difficult and time-consuming task, and perhaps overwhelming for typical consumers. In one instance, there was an abundance of materials directly relevant to twin pregnancies on the home page while for the others, it was either hard to find or not in a form that would be readily understood by an average couple. Others simply stated that the test was available for both singleton and twin pregnancies with little or no supporting information. What was presented appeared to be obscured by a marketing rather than an educational slant, possibly leaving women with a false sense of security. None addressed issues relating to diagnostic testing or the complexity of pregnancy reduction in multiple pregnancies and few provided estimates of screening performance or test failure rates. Academic screening laboratories have historically provided more purely educational materials. Unfortunately, both websites of academic laboratories referred readers to commercial sites for more information. We were unable to find educational materials, readable at an 8<sup>th</sup> grade level or otherwise, that indicated that key issues of value to couples with multifetal pregnancies were being meaningfully addressed in any of these sites.

Ideally, laboratories would provide equivalent types of information for twin pregnancies as for singleton pregnancies including how they assign prior risks and provide detection, false positive, and failure rates along with positive predictive values. They should also include what is known and not known about screening in twins and higher order multiples for couples to make informed decisions. Providers have a responsibility to anticipate questions that may be raised

as a result of the screening process and make couples aware prior to their decisions to be screened via cfDNA. Otherwise, couples will naturally assume there to be no difference in performance or in follow-up testing and future decision-making.

### *Summary of Findings*

1. Prevalence: Rates of twin and triplet pregnancies are currently increased over the natural rate by 1.5 and 5 times, respectively. IVF is the major factor driving this increase that usually results in dizygotic twins. Recent changes in IVF techniques/protocols are resulting in some lowering of these rates. (Moderate Quality)
2. Prevalence: At a given maternal age, rates for common trisomies at the time of screening appear to be similar in twins and singleton pregnancies. (Moderate Quality)
3. Diagnostic testing: CVS and amniocentesis procedures in multiple pregnancies are reliable and safe when performed by a provider experienced in these situations; subsequent diagnostic tests are highly reliable. (Moderate Quality)
4. Professional society statements: Three of 10 statements allow or recommend cfDNA screening in twin pregnancies. Others did not address the issue, suggested data are too sparse, or recommended against. None recommend for, or suggest cfDNA screening in triplet pregnancies might be possible. (High Quality)
5. Traditional screening in twin pregnancies: Maternal age and nuchal translucency (with or without biochemistry) detects up to 80% of Down syndrome at a 5% false positive rate. (Low Quality)

6. cfDNA methods: cfDNA based screening for common trisomies in twins provides higher positive predictive values among twin pregnancies compared with traditional serum and nuchal translucency based screening in twins, but are associated with test failures. (Moderate Quality)
7. cfDNA methods: Although there are several cfDNA testing methodologies available for twin pregnancies, their screening performances appear comparable. (Low Quality)
8. cfDNA methods: Interpretation of the cfDNA test results could differ depending on test methodology, fetal fraction and chorionicity/zygosity. (High Quality)
9. Fetal fraction measurements: Fetal fractions are higher in twin pregnancies, but lower for individual fetuses when compared to singletons. Fetal fractions are correlated between dizygous twins, but can still vary two-fold. (Moderate Quality)
10. Fetal fraction measurements: Multiple methodologies exist that are likely to be internally consistent but no standard is available for harmonization between laboratories. (High Quality)
11. Failure rates for cfDNA testing: Rates in twins range from 1.6% to 13.2%, with a median of 3.6%, higher than for singletons. The median success rate on redraw is about 50% (range 14.3% to 83.3%). IVF and maternal obesity are common risk factors and alternatives include measuring the nuchal translucency. (Moderate Quality)
12. Triplets and cfDNA screening: There are currently few if any observed data to determine detection rates and this is not likely to change in the future. Failure rates up to 20% have been reported. However, based on general knowledge of cfDNA testing, if the fetal fractions

are deemed sufficient and testing is successful, screening performance may approach that found in twin pregnancies. (Low Quality)

13. Patient educational materials: Existing materials for twins may be hard for couples to find, difficult to read and understand, lack information about key performance metrics and fail to distinguish how screening, diagnosis and pregnancy terminations differ from that for singleton pregnancies. (Moderate Quality)

Summary of *evidence-based practices*:

1. The use of first trimester cfDNA screening for the common autosomal trisomies is appropriate for twin pregnancies due to sufficient evidence showing high detection and low false positive rates with high predictive values. Moderate
2. The finding of an increased risk on a cfDNA screening test in multiple pregnancies should be followed by counseling and an offer of diagnostic testing to confirm results. Strong
3. It is preferable for laboratories performing cfDNA testing in multifetal pregnancies to take evidence of zygosity (e.g., chorionicity, sex of the fetuses, embryo transfer history) for the interpretation of both test results and fetal fractions. Moderate
4. When a cfDNA test failure occurs consider ultrasound and diagnostic testing. If there is sufficient time, a second sample draw may also be considered. Moderate
5. Screening options for triplet pregnancies are lacking and cfDNA may be a potential option. However, diagnostic testing should always be offered and the limitations of screening tests stressed. Low



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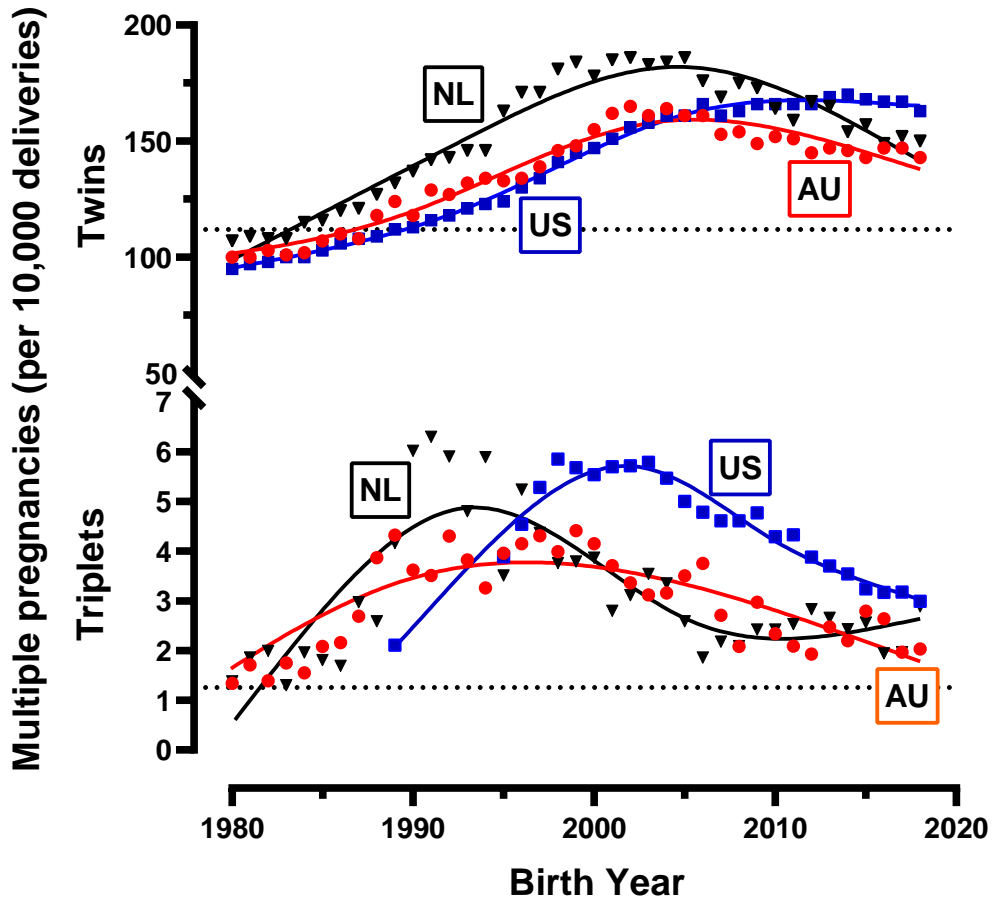
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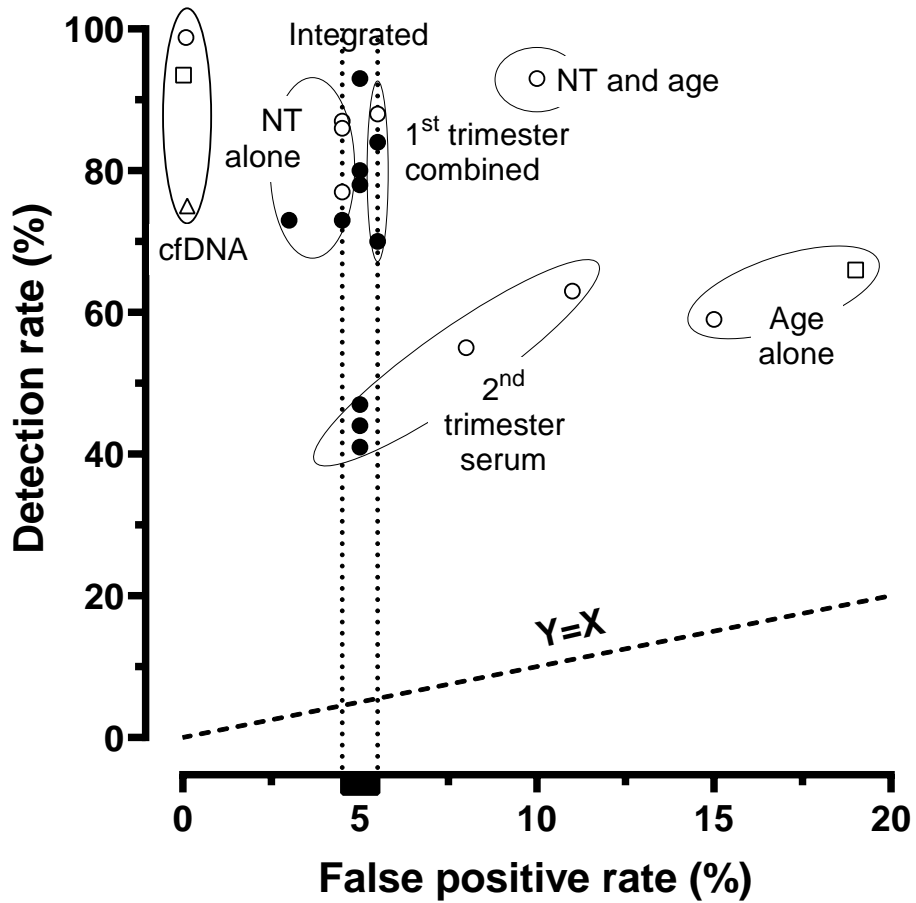


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**Figure 1. Rate per 10,000 of twin and triplet deliveries in three countries from 1980 through 2018.** The horizontal axis shows the year of birth while the split vertical axis shows the rates of triplet deliveries on the bottom and twin deliveries on the top. The horizontal dotted lines indicate the expected rates based on Hellin's Law. These observations are from Australia (red, circles), the Netherlands (black triangles) and the United States (blue squares). Spline curves are shown for each. The maximum rates for twin pregnancies were 158, 181 and 167 per 10,000 and occurred in 2005, 2004 and 2013, respectively. The maximum rates for triplets (base on fitted curves) were 3.7, 4.8 and 5.7 per 10,000, and occurred in 1993, 1994 and 2002, respectively. Data for triplets from both Australia and the Netherlands include a small proportion of higher order multiple pregnancies.



**Figure 2. Twin Pregnancies: Receiver Operator Characteristic (ROC) Curve showing published point estimates of various Down syndrome serum/ultrasound screening combinations in the first trimester, second trimester and in both the first and second trimester.** The horizontal axis displays the false positive rate while the vertical axis displays the corresponding Down syndrome detection (sensitivity) rate. Circles, squares and triangles indicate performance of identifying Down syndrome, trisomy 18 and trisomy 13, respectively. Open symbols show observed performance while the filled circles show modeled performance. Many of the data points are located at a 5% false positive rate (1-specificity), and the vertical dotted lines all indicate that same rate. This is done in order to avoid over-lapping test performance estimates. The straight dashed line ( $Y=X$ ) indicates a 'useless' screening test.

**Table 1. Professional Society Recommendations: cfDNA screening for common aneuploidies in multiple pregnancies**

Year	Society	Twins	Triplets
2019	DEGUM, OGUM, SGUM & FMF Germany <sup>41</sup>	Screening for Down syndrome is similar to that in singletons; data for T18/13 is not yet reliable	Silent on cfDNA testing
2018	HGSA & RANZCOG <sup>42</sup>	May be offered; higher test failure rates and less performance data compared with singletons	Recommends against cfDNA testing Screen with 1 <sup>st</sup> trimester ultrasound (e.g., NT and NB at 11-13 weeks)
2017	SOGC & CCMG <sup>38</sup>	Undertake with caution, less performance data available compared with singletons	Silent on cfDNA testing
2017	PGS & PHGS <sup>43</sup>	Largely silent	Recommends against cfDNA testing
2017	ISUOG <sup>44</sup>	Accuracy of cfDNA testing in twin pregnancies should be investigated further	Silent on cfDNA testing
2016	ACMG <sup>45</sup>	Largely silent. Recommends contacting testing laboratory for multiple pregnancies	Silent on cfDNA testing
2016	ACOG & SMFM <sup>46</sup>	Not recommended because of limited evidence	Not applicable
2016	ESHG ASHG <sup>47</sup>	Silent on cfDNA testing	Silent on cfDNA testing
2016	SFOG <sup>48</sup>	Scientific evidence in multiple pregnancies is insufficient, offer after careful consideration	
2015	ISPD <sup>49</sup>	Similar performance to singletons, if results are interpretable	Risks should be based on ultrasound markers alone; silent on cfDNA testing

DEGUM = German Society of Ultrasound in Medicine and Biology, OGUM = Austrian Society of Ultrasound in Medicine, SGUM = Swiss Society of Ultrasound in Medicine, FMF = Fetal Medicine Foundation, HGSA = Human Genetics Society of Australasia, RANZCOG = Royal Australian and New Zealand College of Obstetricians and Gynaecologists, SOGC = Society of Obstetricians and Gynaecologists of Canada, CCMG = Canadian College of Medical Geneticists, PGS = Polish Gynecological Society, PHGS = Polish Human Genetics Society, ISUOG = International Society of Ultrasound in Obstetrics and Gynecology, ACMG = American College of Medical Genetics and Genomics, ACOG = American College of Obstetricians and Gynecologists, SMFM = Society for Maternal Fetal Medicine, SFOG = Swedish Society of Obstetrics and Gynecology, ISPD = International Society of Prenatal Diagnosis

NT = nuchal translucency, NB = nasal bone

**Table 2. Summary of the detection, false positive and false negative rates for cfDNA testing in twin pregnancies from the published literature.**

Source	N	Trisomy 21			Trisomy 18			Trisomy 13			FPR	
		TP	FN	FP	TP	FN	FP	TP	FN	FP	Tot FP	TN
		DR	FPR		DR	FPR		DR	FPR		FPR (all)	
Gil 2019 <sup>56</sup>	3,780	55	1	2	18	2	1	2	1	6	9	3,688
			98.2%	0.05%		90.0%	0.03%		66.7%	0.19%	0.29%	
Other <sup>53,55,58-63</sup>	851	24	0	2	11	0	0	1	0	0	2	815
			100%	0.25%		100%	0.00%		100%	0.00%	0.24%	
<b>All</b>	<b>4,631</b>	<b>79</b>	<b>1</b>	<b>4</b>	<b>29</b>	<b>2</b>	<b>1</b>	<b>3</b>	<b>1</b>	<b>6</b>	<b>11</b>	<b>4,503</b>
			<b>98.8%</b>	<b>0.09%</b>		<b>93.5%</b>	<b>0.02%</b>		<b>75.0%</b>	<b>0.13%</b>	<b>0.24%</b>	

FPR = False positive rate (1-specificity), N = number of pregnancies, TP = true positive, FN = false negative, FP = false positive, TN = true negative, DR = detection rate (sensitivity)

Further information on individual study results can be found in the Supplemental Materials sTable 1.

**Table 3. Failure / no call rates and fetal fractions among twin and triplet pregnancies**

Study	Method	Twins (Singletons)	No call % (N, rule)	FF Method	Twin FF values <sup>†</sup>	GA <sup>†</sup> (weeks)
Gromminger 2014 <sup>60</sup>	WG	38	13.2% (5, FF < 8%)	Methylation	14.8% (5.4% to 24.8%)	14.3 (9.4 - 23)
Bevilacqua 2015 <sup>71</sup>	Targeted	515 (1,847)	Twins: 5.6% (29) 1 <sup>st</sup> draw [50% (13 / 26) of 2 <sup>nd</sup> draw] <sup>‡</sup> Singletons: 1.7% (32) 1 <sup>st</sup> draw [68% (17 / 28) 2 <sup>nd</sup> draw]	SNPs	Twin with lower FF: 8.7% (4.1 to 30) Singletons: 11% (4.0 to 39)	13.0 (10 - 28) 13.6 (10 - 35)
Sarno 2016 <sup>72</sup>	Targeted	438 (10,698)	Twins: 9.4% (41) 1 <sup>st</sup> draw [51.% (20 / 39) 2 <sup>nd</sup> draw] Singletons: 2.9% (316) 1 <sup>st</sup> draw 63% (148 / 235) 2 <sup>nd</sup> draw	SNPs	MZ (total): 10.1% (IQR 7.6 to 14.5) DZ (lower twin): 7.7% (IQR 5.8 to 10.0) Singletons: 11% (IQR 8.3 to 14)	11.7 (IQR 10.4 - 13) 11.9 (IQR 10.6 to 13)
Tan 2016 <sup>66</sup>	WG	565	3.2% (18) 1 <sup>st</sup> draw [67% (10 / 15) 2 <sup>nd</sup> draw]	Chr Y	8.9% <sup>§</sup> (SD 4.2%) at least one male fetus	12.0 (11 to 28)
Fosler 2017 <sup>61</sup>	WG	487	1.6% (8) 1 <sup>st</sup> draw (cancelled)	Chr Y	One twin: 7.8% <sup>§</sup> (0.8 to 17) Both twins: 16% <sup>§</sup> (2.8 to 32)	13.7 (9.0 to 32.0)
Le Conte 2018 <sup>57</sup>	WG	492	2.4% (12) 1 <sup>st</sup> draw [83.3% (10 / 12) 2 <sup>nd</sup> draw]	Sequence counts	Total: 13.4% (4.6 to 30)	16.3 (10.2 to 35.5)



**Table 3. (continued)**

Chen 2019 <sup>63</sup>	WG <sup>†</sup>	69 twins 85 triplets	Twins: 2.9% (2, FF < 8%) Triplets: 16.5% (14, FF <12%)	cfDNA length	Twins (total): 17.4% <sup>§</sup> (7.5 to 35) Triplets (total): 17.6% <sup>§</sup> (8.9 to 41) Singletons: 11% (4.3 to 22)	Twins: 20 <sup>§</sup> (12 to 30) Triplets: 13 <sup>§</sup> (11 to 19) Singletons: 17 <sup>§</sup> (12 to 30)
Norwitz 2019 <sup>55</sup>	TSNP	117	2.1% had low FF 10.6% No result	SNPs	MZ (twin): 13.0% <sup>§</sup> (SD 4.5%) DZ: (lower twin): 6.5% <sup>§</sup> (SD 3.1%)	NR
Galeva 2019 <sup>54</sup>	Target	928	10.5% (97) 1 <sup>st</sup> draw [57% (50/87) 2 <sup>nd</sup> draw]	SNPs	Not reported. FF included values for MZ (total) and DZ (lower twin)	NR (10,0 to 14,1)
Dyr 2019 <sup>70</sup>	WG	23,986 twins 709 triplets	Twins: 6.0% (1,313 low FF) Triplets: 21.3% (146 low FF)	Methylation / Sequence counts	Twins (total): 12.3% <sup>§</sup> Triplets (total): 13.2% <sup>§</sup>	Twins: 13.7 <sup>§</sup> (9 to 38) Triplets: 13.1 <sup>§</sup> (9 to 33)

FF= fetal fraction, GA = gestational age, WG = whole genome, SNPs = single nucleotide polymorphisms, IQR = interquartile range, SD = standard deviation, MZ = monozygous, DZ = dizygous, TSNP = targeted single nucleotide polymorphisms, NR = not reported

† Fetal fraction (FF) and gestational age (GA) are medians and ranges, unless otherwise indicated

‡ Success rates of 2<sup>nd</sup> blood draws are shown in square brackets.

§ average

†† Paired end sequencing



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