Confirmation rate of cell free DNA screening for sex chromosomal abnormalities according to the method of confirmatory testing

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Abstract
Objective: To examine the positive predictive value (PPV) of cfDNA screening for sex chromosome aneuploidies (SCA) in a large series of over 90 000 patients.
Methods: Retrospective study based on samples that were sent to Cenata, a private laboratory which uses the Harmony Prenatal Test. The SCA high-risk results were stratified according to the method of diagnostic testing and according to karyotype result.
Results: The study population consisted of 144 cases. The CfDNA test indicated monosomy X, XXX, XXY, and XYY in 62, 37, 40, and 5 cases, respectively. The overall PPV was 38.9% (30.9-47.4), 29.0% (18.2-42.9) for monosomy X, 29.7% (15.9-47.9) for 47,XXX, 57.5% (40.9-73.0) for 47,XXY, and 80.0% (28.4-99.5) for 47,XYY. A total of 112 (77.8%) women with a high-risk result for SCAs opted for prenatal karyotyping. In this group, there were significant differences in the PPV if the karyotype was assessed by amniocentesis or by CVS: 29.5% vs 50.0%. This significant difference was driven by the monosomy X result which shows a significantly higher PPV in CVS (54.6% (23.4-83.3) vs 17.1% (6.6-33.6)). For the other SCAs, the differences were not significant.
Conclusion: PPV of an abnormal cfDNA test for SCAs is low, particularly for monosomy X. The confirmation rate depends on the type of confirmatory test.

1 INTRODUCTION

Cell free DNA (cfDNA) screening for common trisomies has become the gold standard in screening for trisomy 21, 18, and 13 with detection rates of around 99% and a false positive rate of 0.1%.1 In several countries this approach has replaced first trimester combined screening, especially if it is combined with a detailed ultrasound examination.2

There is an ongoing discussion whether the scope of the cfDNA analysis should be expanded to cover other genetic diseases.3-7 CfDNA tests are already available on commercial basis for sex chromosomal defects, genome-wide imbalances (eg, rare autosomal trisomies and large segmental imbalances), some recurrent microdeletions, and a few monogenetic diseases. However, several scientific societies argue that the disadvantages that may evolve from extended genetic testing could outweigh the benefits.4,8-10

While implementation modalities for screening for chromosomal defects other than common trisomies are still in development, the translation into clinical practice of screening for sex chromosome aneuploidies (SCAs) has quickly evolved and now it is a well-established investigation in concomitance with that for trisomy 21, 18, and 13. This involves screening for monosomy X (MX), triple X (XXX), XYY, and XXY syndromes that cumulatively have an incidence of 1/350 to 1/400 live births and, after trisomy 21, are the most frequent diagnosis at the time of amniocentesis.11-13 Despite the rapid technological advancement, only few studies have examined the
effectiveness of cfDNA screening for SCAs. In a meta-analysis of Gil et al, the detection rate for sex chromosomal defects was between 96% and 100% for a false positive rate between 0.004% and 0.14%. In total, the meta-analysis included 36 fetuses with monosomy X and 15 fetuses with other sex chromosomal defects from studies with a follow-up available in 85% of tested pregnancies. Genetic follow-up in studies that focus on SCA is particularly important as 47,XXY, 47,XXX, 47,XYY have relatively few serious physical abnormalities, the phenotypic features are extremely variable. Therefore they may go undetected at birth or even at later ages.

In this study, we set out to examine the positive predictive value (PPV) of cfDNA screening for sex chromosomal abnormalities in a large series of over 90 000 patients.

2 | METHODS

This retrospective study is based on samples that were examined by Cenata, a private laboratory for prenatal noninvasive testing in Germany which uses Harmony Prenatal Test (Roche, Inc., San Jose, California) as previously described. In brief, this is a targeted cfDNA analysis using microarray quantitation of DANSR (Digital ANalysis of Selected Regions) assays of non-polymorphic (chromosomes 13, 18, 21, X, and Y) and polymorphic loci (chromosomes 1-12) to estimate chromosome proportion and fetal fraction, respectively. The FORTE (Fetal fraction Optimized Risk of Trisomy Evaluation) algorithm is used to provide patient-specific risk assessments. A risk score of greater than 1% (1 in 100) is considered a high-risk result. The cohort comprised of samples from women of at least 18 years of age with singleton pregnancies. The gestational age at the time of blood sampling was at least 11 weeks' gestation. Blood was collected and sent in two 10 mL blood collection tubes containing a stabilizer for cell free DNA. The samples were stored at room temperature and sent within 7 days after collection directly to Cenata without prior processing.

Samples were received from several countries. All women were counseled prior to the cfDNA analysis and after testing according to the national regulations. In cases with an abnormal cfDNA result, the pregnant women were counseled by their obstetrician or by a clinical geneticist. Further management including invasive testing and genetic follow-up examinations were in line with the national guidelines. In general, this involved conventional karyotyping. In cases with a CVS, only the long-term culture was used for this study. Genetic follow-up examinations of mosaic or fully abnormal cases on mesenchyme, to distinguish between fetal and placental pathology, was not available.

In the informed consent, all women are asked if they or their obstetricians can be contacted to obtain the outcome of the pregnancy and all relevant information regarding the test provision. This is part of the routine laboratory quality assessment program of Cenata. Only women who gave their written consent and for whom the outcome data were available are involved in this study.

Approval for the study was obtained from the ethical committee of the university hospital of Tübingen (No. 304/2020BO2).

What’s already known about this topic?
- The positive predictive value of prenatal cfDNA screening for sex chromosomal abnormalities is lower than for common trisomies.

What does this study add?
- This study demonstrates how the confirmation rates differ depending on the method of the confirmatory test.

2.1 | Statistical analysis

The cfDNA test results were stratified according to the method of diagnostic testing and according to karyotype. We classified a cfDNA result as being confirmed if the karyotype on amniocytes or on the newborn's blood was abnormal with the same abnormality pointed out by the cfDNA test either in a homogeneous or mosaic form. If the fetal karyotype was normal or abnormal but different from the cfDNA result indicating a SCA, the case was classified as not confirmed. The PPV was calculated as proportion of abnormal cases with a concordantly abnormal test result divided through all cases with the abnormal test result.

The data are presented as median with 25 to 75th interquartile range, or as percentage, whenever appropriate. Proportions are compared with a Chi-square test. The P-level of .05 was considered as threshold for a significant difference. Ninety-five percentage confidence intervals were computed according to the methods of Clopper and Pearson.

3 | RESULTS

Between October 2017 and June 2019, 93 048 cfDNA tests were carried out by Cenata. In 70 449 (75.7%) cases, an assessment of the SCAs was requested and in 66 203 (94.0%) of these cases, a test result was reported. In 4246 cases (5.8%), it was not possible to report on SCAs either because the cfDNA test failed completely (4.2%) or because only the SCA analysis (1.6%) failed. These values represent the final non-informativity rate.

In 351 (0.53%) pregnancies, the cfDNA screening indicated a SCA. Within this group, 294 (83.8%) women agreed to share information regarding the outcome of the pregnancy, however 150 (51.0%) of them opted against prenatal or postnatal genetic testing. These pregnancies were excluded from further analysis. Thus, our study population consisted of 144 cases with a confirmatory diagnostic (invasive or postnatal) procedure.

Median maternal and gestational age was 35.4 (IQR 31.1-37.5) and 12.6 (IQR 11.4-13.4) weeks, respectively. Median fetal fraction was 8.0% (IQR 5.9-11.3). For comparison, the fetal fraction was 10.6% (IQR 8.2-13.4) in the euploid population.

approval for the study was obtained from the ethical committee of the university hospital of Tübingen (No. 304/2020BO2).
An amniocentesis was carried out in 78 and a CVS in 34 cases. Postnatal testing was performed in 32 pregnancies (Table 1).

In the group of pregnancies where a confirmatory (antenatal or postnatal) karyotype was available, cfDNA analysis indicated MX in 62 (43.1%) cases, XXX, XXY, and XYY karyotypes were seen in 37 (25.7%), 40 (27.8%), and 5 (2.8%) cases, respectively.

In total, the cfDNA test result was confirmed in 56 (38.9%) cases. A total of 112 (77.8%) women with a positive result for SCAs opted for an antenatal invasive procedure. Among them, there were marked differences in the PPV if the karyotype was assessed by amniocentesis or by CVS. In the first group, the cfDNA and amniocentesis result was consistent in 17 (50.0%) of the 34 cases, respectively (Chi-square test \( P = 0.037 \)). The significant difference between the confirmation rate by CVS and amniocentesis was predominantly driven by the MX result (54.6 vs 17.1%, respectively, \( P = 0.014 \)). For the other SCAs, the differences were not significant. The confirmation rates stratified by type of SCA identified by the cfDNA test and by diagnostic confirmatory procedure are shown in Table 1.

In three cases where the cfDNA analysis reported a high-risk result for MX (\( n = 1 \)) or XXX (\( n = 2 \)), the karyotype on amniocytes was abnormal but discordant with the cfDNA test result. In one of the cases where the cfDNA indicated XXX, the karyotype was mosaic trisomy 13. In the other XXX case the actual karyotype was 46,XX,dup(X)(q21.31q25)d. In the case with a high-risk result for MX, the karyotype was 47,XXY.

4 | DISCUSSION

In this study, we have examined the PPV of an abnormal cfDNA test result indicating a SCA with the DANSR technology. Our study has shown that overall, in only 39% of cases the suspected SCA was confirmed by pre- or postnatal karyotyping. In addition, there was a significant difference in the confirmation rate depending on the type of confirmatory invasive testing and on the type of SCA. The overall PPV for MX and XXX was similar at about 29% which was much lower than the PPV for XXY and XYY.

In the amniocentesis group, the overall confirmation rate was only 30%, compared to 50% in the CVS group. The difference was even more pronounced in cases with a suspected MX where the confirmation rates were 17% and 55%, respectively. One possible explanation is the absence of genetic follow-up examinations in case of an abnormal mesenchyme result in order to distinguish between fetal and placental pathology. Furthermore, the absence of an extended analysis on the cytotrophoblast in combination with the mesenchyme may have also contributed to the difference between CVS and amniocentesis. In about 15% of the cases with a fully abnormal SCA result in the mesenchyme, the respective amniocytes will be normal.

In addition, the detection of a mosaic in the mesenchyme is not indicative of a fetal abnormality. Therefore, these findings should always be followed up by a subsequent confirmatory amniocentesis. Unfortunately, in this study, it was not possible to collect this information.

Another explanation for the higher confirmation rate of MX in CVS may be that the choice for a CVS was probably driven by an abnormal ultrasound examination. For these reasons, we believe, that the PPVs on CVS probably may be overestimated. On the other hand, in most of the cases in our study, an amniocentesis or newborn blood was investigated, which gives more reliable results.

There are well known technical, statistical, and biological reasons for discordant results between the cfDNA screening result and the real fetal karyotype.

### TABLE 1 Positive predictive value (PPV) stratified by type of SCA identified by cfDNA test and by diagnostic confirmatory procedure. The percentages represent the positive predictive value

<table>
<thead>
<tr>
<th>cfDNA result</th>
<th>Total n</th>
<th>Confirmed n PPV % (95% CI)</th>
<th>CVS n</th>
<th>Confirmed n PPV % (95% CI)</th>
<th>Amniocentesis n</th>
<th>Confirmed n PPV % (95% CI)</th>
<th>Newborn’s blood n</th>
<th>Confirmed n PPV % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX</td>
<td>62</td>
<td>18 (29.0 (18.2-42.0))</td>
<td>11</td>
<td>6* (54.6 (23.4-83.3))</td>
<td>35</td>
<td>6* (17.1 (6.6-33.6))</td>
<td>16</td>
<td>6 (37.5 (15.2-64.6))</td>
</tr>
<tr>
<td>XXX</td>
<td>37</td>
<td>11 (29.7 (15.9-47.0))</td>
<td>8</td>
<td>2** (25.0 (3.2-65.1))</td>
<td>25</td>
<td>8** (32.0 (14.9-53.5))</td>
<td>4</td>
<td>1 (25.0 (0.6-80.6))</td>
</tr>
<tr>
<td>XXY</td>
<td>40</td>
<td>23 (57.5 (40.9-73.0))</td>
<td>14</td>
<td>8*** (57.1 (28.9-82.3))</td>
<td>15</td>
<td>7*** (46.7 (21.3-73.4))</td>
<td>11</td>
<td>8 (72.7 (39.0-94.0))</td>
</tr>
<tr>
<td>XYY</td>
<td>5</td>
<td>4 (80.0 (28.4-99.5))</td>
<td>1</td>
<td>1**** (100 (2.5-100))</td>
<td>3</td>
<td>2**** (66.0 (9.4-99.2))</td>
<td>1</td>
<td>1 (100 (2.5-100))</td>
</tr>
<tr>
<td>TOTAL</td>
<td>144</td>
<td>56 (38.9 (30.9-47.4))</td>
<td>34</td>
<td>17**** (50.0 (32.4-67.7))</td>
<td>78</td>
<td>23**** (29.5 (19.7-40.9))</td>
<td>32</td>
<td>16 (50.0 (31.9-68.1))</td>
</tr>
</tbody>
</table>

Note: Chi-square tests between confirmed cases by amniocentesis vs CVS.

* \( P = .014 \).
** \( P = .708 \).
*** \( P = .573 \).
**** \( P = .505 \).
***** \( P = .037 \).
Biological reasons for discordant results, especially for MX, are a former dichorionic twin pregnancy with a vanishing twin, maternal constitutional mosaicism, rarely an undetected maternal tumor, and the spontaneous loss of an X chromosome in women's lymphocytes during ageing.11,19-22

The main biological reasons for discordant results however are fetoplacental mosaicism. Cell free DNA in the maternal blood that can be allocated to the pregnancy is produced by the apoptosis of the trophoblast layer of the chorionic villi and not by the fetus itself. The direct preparation of CVS and the cfDNA screening test are therefore based on the same cell lineage, the cytotrophoblast and thus are both prone to the same potential biological confounding factors, such as fetoplacental mosaicism. Nearly 2% of the pregnancies are affected by such biological phenomena, whereby the cytotrophoblast genetic constitution may not match that of the fetus.23,17 In these situations, the cfDNA test may provide a false positive or negative result when compared to the fetal karyotype on amniocytes, the latter being the reference standard, as it represents the genetic constitution of cells derived from different fetal organs and tissues.24

An interesting example for fetoplacental discordance and placental mosaicism was shown by our workgroup.25 In a pregnancy with fetus.23,17 In these situations, the cfDNA test may provide a false positive or negative result when compared to the fetal karyotype on amniocytes, the latter being the reference standard, as it represents the genetic constitution of cells derived from different fetal organs and tissues.24

An interesting example for fetoplacental discordance and placental mosaicism was shown by our workgroup.25 In a pregnancy with MX high-risk result, the follow-up karyotype on four different sites of the term placenta and on amniocytes showed a complex puzzle of different cell lines: the fetus and the placental site 1 showed a 46,X,i(iddY)(q11.21) karyotype, while in the other sites, there was a mixture of different levels of mosaicism with cells having either a 45,X or a 46,X,i(iddY)(q11.21) karyotype.

Based on the prevalence of discordant results between cytotrophoblast by CVS direct preparation and amniocytes, the test performance of cfDNA screening for MX is projected to be worse than for common trisomies.23,24 This is not surprising, given the fact that MX is the chromosome abnormality that is most often involved in fetoplacental mosaicism.23

If a CVS is carried out after an abnormal cfDNA screening test for trisomy 21, 18, and 13, a chromosomal mosaicism in the placenta will be found in 2%, 4%, and 22% of cases, respectively, compared to 59% of cases where the test indicates MX.21 However, if the MX high-risk result is accompanied by a fetal anomaly, the incidence of placental mosaicism is expected to be reduced to about 2%.21,26 Therefore it should be emphasized that the method of choice for investigating the fetal karyotype is amniocentesis and not CVS in cases where the cfDNA indicates an SCA and the fetal anatomy is normal.26 This is particularly true for MX. The remaining SCAs (XXX, XXY, XYY) are less prone to fetoplacental mosaicism. In addition, especially SCAs involving the Y chromosome are less susceptible to other confounding factors.26 Therefore, a CVS can be an option under the caveat of a likelihood of mosaic detection <10%.26

Our results are partly in line with previous studies. As many studies have only included few abnormal cases, we have focused on those that describe the outcome of at least 10 cases with MX and in which the assessment risk for all SCAs is performed.

Previous study results are summarized in Table 2. All studies indicated that the PPV for cfDNA indicating SCAs, particularly for MX, is lower than for common trisomies.

In a large retrospective study from a genetic diagnostic laboratory, the authors estimated the PPV of cfDNA screening for SCA on 124 high-risk cases. For MX it was 27% and for XXX and XXY syndrome 45% and 85%, respectively. The number of cases with a positive cfDNA screen for XYY was too low to accurately calculate the PPV (only four cases).33

### Table 2

<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>MX</th>
<th>XXY</th>
<th>XXX</th>
<th>XYY</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zheng et al27</td>
<td>MPS</td>
<td>2/14</td>
<td>5/10</td>
<td>6/9</td>
<td>5/7</td>
<td>18/40</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>14.3%</td>
<td>50%</td>
<td>66.7%</td>
<td>71.4%</td>
<td>45%</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>20%</td>
<td>50%</td>
<td>33.3%</td>
<td>100%</td>
<td>34.7%</td>
</tr>
<tr>
<td>Wang et al29</td>
<td>MPS</td>
<td>3/14</td>
<td>10/11</td>
<td>3/4</td>
<td>3/4</td>
<td>19/33</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>21.4%</td>
<td>90.9%</td>
<td>75%</td>
<td>75%</td>
<td>57.6%</td>
</tr>
<tr>
<td>Zhang et al30</td>
<td>MPS</td>
<td>5/17</td>
<td>7/9</td>
<td>5/5</td>
<td>1/1</td>
<td>18/32</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>29.4%</td>
<td>77.8%</td>
<td>100%</td>
<td>100%</td>
<td>56.3%</td>
</tr>
<tr>
<td>Suo et al31</td>
<td>MPS</td>
<td>12/37</td>
<td>6/10</td>
<td>5/9</td>
<td>7/8</td>
<td>30/64</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>32.4%</td>
<td>60%</td>
<td>55.6%</td>
<td>87.5%</td>
<td>46.9%</td>
</tr>
<tr>
<td>Garshasbi et al32</td>
<td>MPS</td>
<td>10/15</td>
<td>4/5</td>
<td>4/6</td>
<td>1/3</td>
<td>19/29</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>66.7%</td>
<td>80%</td>
<td>66.7%</td>
<td>33.3%</td>
<td>65.5%</td>
</tr>
<tr>
<td>Our study</td>
<td>DANSR</td>
<td>18/62</td>
<td>23/40</td>
<td>11/37</td>
<td>4/5</td>
<td>56/144</td>
</tr>
<tr>
<td></td>
<td>PPV</td>
<td>29%</td>
<td>57.5%</td>
<td>29.7%</td>
<td>80%</td>
<td>38.9%</td>
</tr>
<tr>
<td>Total</td>
<td>PPV</td>
<td>55/184</td>
<td>61/97</td>
<td>37/79</td>
<td>24/31</td>
<td>7/121119</td>
</tr>
<tr>
<td></td>
<td>FPR</td>
<td>29.9%</td>
<td>62.9%</td>
<td>46.8%</td>
<td>77.4%</td>
<td>0.01%</td>
</tr>
</tbody>
</table>
In three previous studies based on DANSR technology applied on maternal plasma samples, the test performance of cfDNA screening for SCAs was examined in 1294 euploid pregnancies and 87 fetuses with MX, 7 with XXX, and 8 with XXY syndrome. The detection and false positive rates were between 91.5%-100% and 0%-0.6%, respectively. In contrast to our study, these were case-control studies based on frozen maternal plasma samples, and therefore aimed to assess the analytical, and not clinical, performances of the test.

For completeness, we have also collected and shown the information regarding the karyotype of the few cases where the follow-up examination of the abnormal cfDNA test was carried out after birth. Half of the cases were true positives, which is an unexpectedly high confirmation rate. We assume that at least some of these newborns were symptomatic and genetic follow-up examinations were prompted by a clinical indication rather than by the abnormal prenatal cfDNA test, hence the high confirmation rate.

The strength of our research lies in the large number of high-risk cases and providing a PPV stratification by type of confirmatory diagnostic procedure. This is relevant to understand the real-life implications of the cfDNA test for SCAs. However, there are also some limitations. Firstly, the confirmatory karyotype on CVS was based on long-term culture only which provides the assessment of mesenchyme alone. Therefore, we might have misclassified as true positives those cases affected by a confined placental mosaicism with an homogeneous SCA on mesenchyme or as false positives those cases affected by a TFM (true fetal mosaicism) for a homogeneous or mosaic SCA with a normal mesenchyme but abnormal cytotrophoblast. Unfortunately, information on additional postnatal genetic follow-up was not available for any of these cases. In addition, it was not possible to gather information regarding the follow-up testing on amniocytes or newborns' blood in cases with a mosaic SCA on mesenchyme. Secondly, only about half of the women with an abnormal cfDNA result indicating a SCA underwent an antenatal invasive testing. The outcome of the other pregnancies was not available. Thirdly, we could not collect karyotype from products of conception. MX is frequently found in spontaneous pregnancy losses/abortions. Therefore, PPV for MX might be underestimated. Finally, we do not have information regarding possible structural fetal defects, increased nuchal translucency thickness or hygroma in the pregnancies which provided the samples examined in our study. We assume that in cases with fetal abnormalities, the confirmation rate would have been higher than in our series.

In summary, our study has shown that, in contrast to common trisomies, the confirmation rate of an abnormal cfDNA screening test for SCAs is lower, particularly for MX. The confirmation rate seems to be depending on the type of confirmatory test performed.

CONFLICT OF INTEREST
Kai Lüthgens, Monika Sinzel, and Karina Haebig work at Cenata GmbH and Francesca Romana Grati at TOMA Advanced Biomedical Assays S.p.A., Impact Lab Group. In both labs, the Harmony test is offered on a commercial basis. Francesca Romana Grati is an advisory board member for Roche and Menarini Biomarkers.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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