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Increasing maternal age is not a significant cause of false positive results for Monosomy X in non-invasive prenatal testing

Running title: False positive results for Monosomy X in NIPT

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What is already known about this topic?

- NIPT can screen for monosomy X during pregnancy
- Monosomy X has the lowest PPV of aneuploidies screened for with NIPT

What does this study add?

- Ultrasound findings and placental biomarkers are strong predictors of a false positive NIPT result
- Pre-test counselling doesn't need to address advanced maternal age when discussing PPV

- Post-test counselling should include consideration of ultrasound findings and placental biomarkers for diagnostic decision making

Data availability: 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.

Introduction

Non-invasive prenatal testing (NIPT) analyses cell-free foetal deoxyribonucleic acid (cfDNA) in maternal blood to screen for the three most common chromosome aneuploidies in pregnancy; trisomy 21, trisomy 18 and trisomy 13[1,2]. NIPT is also able to assess the sex chromosomes in order to screen for sex chromosome aneuploidies (SCA) as well as determine foetal sex[3]. The four SCA assessed include 45,X (monosomy X [MX] or Turner syndrome), 47,XXX (triple X syndrome), 47,XXY (Klinefelter syndrome), and 47,XYY (Jacob syndrome). Unlike autosomal trisomies, MX appears to have an inverse relationship with maternal age[4,5]. A clinical diagnosis of Turner syndrome is given to approximately 1 in 1,800 live births, however it is estimated that the incidence of MX conceptions is much higher with up to 98% of MX conceptions ending in early spontaneous miscarriage[6,7,8]. Recent studies and meta-analyses have demonstrated that the positive predictive value (PPV) for NIPT varies depending on the chromosome assessed[9,10]. Monosomy X (MX) has the lowest PPV of all aneuploidies (19% to 29%) whilst the highest PPV is for trisomy 21 (83% to 91%)[10,11,12,13].

There are several biological explanations for a false positive NIPT result. These include confined placental mosaicism, demise of a co-twin with aneuploidy, maternal copy number variation, maternal mosaicism or maternal malignancy[2,14]. To assist with post-test counselling and monitoring for possible discordant results, attention should also be paid to other factors such as ultrasound findings and foetal fraction (FF)[15]. Additionally, when an

increased nuchal thickness is detected on ultrasound, NIPT can correctly identify one of the common aneuploidies or MX in up to 95% of cases[16].

These biological explanations apply to all aneuploidies assessed by cfDNA and are not unique to MX, thus do not entirely account for the low PPV in MX high risk cases. One possible biological factor that is specific to MX false positive results is the phenomenon of X-inactivation. This is a process which occurs in female somatic cells in order to account for the imbalance of X chromosome material between males and females[17]. A consequence of X-inactivation is increasing X chromosome loss with increasing age which can resemble low level MX mosaicism in blood and buccal samples [18,19]. As NIPT analyses both maternal cfDNA and foetal cfDNA, changes to the maternal cfDNA can erroneously be attributed by the NIPT software to the foetus. A recent study reported a statistically significant correlation between higher maternal age and higher false positive rates for MX[14]. The aim of this study is to investigate maternal age and other possible predictors of false positive MX results on NIPT in order to guide pre-test and post-test counselling.

Methods

Participants & setting

This retrospective cohort study identified NIPT results, demographic data, and diagnostic testing results for patients who attended one of three private specialist prenatal screening practices in Sydney and Melbourne, Australia, from March 2013 to December 2018. NIPT was undertaken as either first-tier or second-tier screening. Ultrasound was conducted prior to NIPT sample collection to confirm an on-going pregnancy of at least 10 weeks' gestation and to determine foetal number. Two of the three practices had an 'opt-in' policy for SCA screening and the other provided SCA assessment on an 'opt-out' basis. Twin and higher order multiple pregnancies were excluded as SCA can only be assessed in singleton pregnancies. There were no other exclusion criteria. Pre-test oral consent was obtained after discussing the accuracy of NIPT for the common trisomies, foetal sex and SCA testing

where appropriate. Consent included obtaining pregnancy outcome information from the referring clinician as necessary.

Procedures & measures

Four NIPT service providers were used over the course of this study, all of which report comparable screening accuracy. Cell-free DNA analysis was performed by a mix of whole genome sequencing, targeted sequencing and microarray-based platforms. Foetal fraction was assessed using two different methods by three of the providers and not reported by the fourth provider for the first half of the study period. Clinical and demographic data obtained for patients who returned a high probability MX result included maternal age, body mass index (BMI) at the time of blood collection, self-reported primary ethnicity, gestational age, NIPT service provider, mode of conception, FF, placental biomarkers, nuchal translucency (NT) measurement and foetal structural assessment where available[20].Availability of placental biomarkers and NT measurement was often dependant on gestation at the time of blood collection, and whether NIPT was elected as first or second tier screening. Data on the type and result of any prenatal or postnatal diagnostic testing or testing of products of conception in the event of miscarriage or pregnancy termination were also sought from the clinic records or from the referring clinicians. Prenatal testing choice was in part determined by the foetal assessment, with amniocentesis preferred in apparently structurally normal foetuses (to reduce potential for confined placental mosaicism inherent in CVS) and CVS more commonly performed when structural anomalies were identified (to facilitate earlier diagnosis). Once all available outcome data were obtained, NIPT results were classified as either true positive (including foetal mosaicism) or false positive. Outcome data were not sought for low probability results.

Statistical analysis

Categorical variables are expressed as numbers and proportions and continuous variables in means and standard deviations (SD) or medians and interquartile ranges (IQR) depending on the frequency distribution. Differences between groups with known and unknown

pregnancy outcomes were assessed with Fisher's exact test for categorical variables and with independent Student t-test or Mann-Whitney U test for continuous variables, as appropriate.

Simple logistic regression was performed to investigate the influence of the independent predictor variables on the occurrence of false-positive MX results. Scatter plots with superimposed lowess curves (a nonparametric estimator of the mean function) were graphed to identify patterns of change in the probability of obtaining a false-positive MX result as a function of these variables. Further analyses were performed for each of the potential explanatory variables using correct classification percentage and area under the curve to further inform dichotomization. Statistical analysis was performed in Stata version 15.1 (StataCorp. 2017, College Station, TX), with α=0.05 used to define statistical significance.

Ethical considerations

As this study is a clinical audit of an established screening programme as identified by the National Health and Medical Research Council (2014)[21], formal approval by a Human Research Ethics Committee was not required.

Results

Participants

Over the 69 months study period, 52,499 cell-free DNA tests were analysed. However, 1,995 (3.8%) samples were from twin pregnancies and were excluded. Sex chromosome analysis was performed on an opt-in basis in two of the three practices, thus a further 3,285 (6.3%) samples with no information on X-chromosome were excluded, leaving a final cohort of 47,219 pregnancies.

There were 107 cases at high risk for monosomy X (1 in 441 pregnancies), of whom nine (8%) patients declined prenatal or postnatal chromosome analysis and a further two (2%)

were lost to follow up. There were no significant differences in maternal age (p=0.26), BMI (p=0.36), gestation at test (p=0.45) or FF (p=0.50) between the 96 cases with known outcomes and the 11 cases where outcome was unknown. The final cohort was composed of these 96 cases, with demographic and clinical characteristics presented in Table 1. Most were Caucasian women (82%) of normal weight (BMI 18 - 25 kg/m^2; 61%) with spontaneous conceptions (86%) undertaking cfDNA testing at a median gestation of 11.0 weeks.

Outcome data

From the 96 screen positive cases, 19 (20%) chose to undertake chorionic villus sampling (CVS), one (1%) had both CVS (mosaic MX) and amniocentesis (46,XX), 55 (57%) had amniocentesis, 15 (16%) were tested postnatally, and six (6%) had confirmation on products of conception following miscarriage or termination of pregnancy with hydrops fetalis.

Ultrasound features suggestive of monosomy X (including nuchal translucency exceeding 3.5mm (the 99th centile at 11-14 weeks), prominent jugulo-lymphatic sacs, cystic hygroma (larger poster-lateral cystic spaces separated by a posterior midline septum), sub-cutaneous oedema or cavity effusions [hydrops fetalis]) occurred in 16 (18%) of the cases with ultrasound information recorded. In the prenatal cases displaying ultrasound features consistent with MX, CVS was significantly more likely to be undertaken than amniocentesis (8/20 [40%] vs. 0/56 [0%]; Fisher's exact test p<0.001). Ultrasound features were also noted in three (20%) of 15 cases tested postnatally and in all six cases where termination of pregnancy or miscarriage occurred.

Bivariable analysis of test outcomes

False positive results occurred in 71 (74%) cases and true positive results were identified in 25 (26%) cases. There was a significant association between confirmatory test type and true positive results with CVS and products of conception having a higher proportion compared with amniocentesis or postnatal testing (56% vs. 16%; Fisher's exact test p<0.001).

Bivariable analysis commenced with visual assessment of scatterplots of explanatory variables against the occurrence of a false positive result and their associated lowess curves (Figure 1). On the basis of the lowess curves and further analyses, explanatory variables were partitioned into categories for logistic regression and effect size assessment. These categories included maternal age (<38 / ≥38 years), BMI (≥25 / <25 kg/m^2), ethnicity (Caucasian / Non-Caucasian), conception method (spontaneous / assisted reproduction) and ultrasound (+/- ultrasound MX features). There was no significant difference in false positives rates between service providers A and B (p=0.73) or between providers C and D (p=0.99) and they were grouped accordingly.

The effect sizes are detailed in Table 2 and depicted in Figure 2. The likelihood of a false positive result increases with advanced maternal age (odds ratio [OR] 1.60), being overweight / obese (OR 2.03), being tested by service provider A or B (OR 4.04), having higher placental serology results (PAPP-A MoM OR 5.97; PlGF MoM OR 3.87) and decreases with Asian ethnicity (OR 0.53) and if any ultrasound features associated with monosomy X are identified (OR 0.02). Although the effect sizes were relatively large, only service provider choice, PAPP-A MoM and monosomy X ultrasound features were statistically significant.

Discussion

This study confirmed the poor predictive capacity of a high risk monosomy X result (PPV < 30%) and showed that the likelihood of a false positive MX result increased with increasing maternal age, increasing BMI, choice of NIPT provider, increasing placental serology levels, Caucasian ethnicity and absence of ultrasound findings suggestive of MX, but only three of these factors were shown to be statistically significant, not including maternal age.

Increased maternal age has previously been reported to be associated with false positive MX results[14], a non-significant trend that was identified in the current study. A plausible biological explanation exists for this in that the proportion of female peripheral blood cells showing X-chromosome loss is less than 1% for women under the age of 25 years, but then begins to increase with age and approaches 2% by 40 years[18]. As the association between maternal age and a MX result from NIPT is weak, it is not necessary for pre-test counselling to include discussion of a possible higher rate of false positive MX result for women of advanced maternal age.

Ultrasound features of MX were significantly associated with a true positive result. This is not a novel finding, however with NIPT becoming an increasingly popular choice for both low and high priori risk populations[22,23], it does highlight the continuing importance of a thorough 11-14 weeks ultrasound examination after cfDNA testing. We also detected a significant association between the type of confirmatory test used and the likelihood of a false positive result. Clinicians at the three practices in this study have been combining ultrasound and placental serology with NIPT result to assist with post-test counselling for several years. Clinical advice would favour earlier prenatal diagnosis by CVS when there are ultrasound findings in addition to a high probability result for MX on NIPT. Cases where products of conception were tested were more likely to be true positive results, which is supported by the high rate of first trimester spontaneous miscarriage in MX conceptions[6]. In the absence of any other findings to support a true positive result, clinicians are more likely to recommend amniocentesis, or patients may even decline PND instead confirming a false

positive result at postnatal testing. Due to the high spontaneous miscarriage rate, viability beyond 15 weeks of gestation, with an opportunity to undertake amniocentesis, is more likely to return a false positive result. Therefore, the finding that confirmatory testing is associated with likelihood of false positive is more of a reflection of clinical practice and natural history of MX than a predictor of PPV.

A low level of PAPP-A was the only placental serology result which showed to be a significant predictor of a true positive result, although a similar trend was also observed in the small number that had PlGF testing. This result may have been influenced by abnormally low levels of PAPP-A from one of the data collection centres, only involving 11 patients, and it was unclear from the records whether there was a selection bias in ordering serology test in the these individuals. Low PAPP-A levels have been previously associated with abnormal karyotype (including MX), foetal growth restriction, spontaneous miscarriage, and pre-eclampsia[24,25,26,27,28]. Service provider choice also proved statistically significant in the correct classification of MX cases. Providers A and B had higher false positive rates for MX than providers C and D. This may be explained by a combination of provider A not measuring FF for approximately the first half of the study period, and provider B using a single nucleotide polymorphism based platform compared to providers A, C and D who all used a whole genome sequencing based platform . This highlights the importance of transparent audit of test accuracy by providers to assist referrers in making clinical testing decisions. No association was identified between FF and false positive result for MX in this study, unlike a previous study relating to discordant results for the common trisomies[15]. This was an unexpected finding given the positive association between BMI and false positive results. However, several other factors are known to affect FF, notably gestational age, and free beta-hCG[29], which could influence PPV. The different FF assessment methodologies used by the providers and missing data may also have contributed to this finding.

Post-test counselling for patients who receive a high probability result for MX should be informed not only by the reported cfDNA test PPV but also by ultrasound findings and

placental biomarker levels to tailor a more accurate individual assessment of the likelihood for a false positive result. If patients elect to undertake prenatal diagnosis an individual assessment combining these factors can assist in counselling between CVS and amniocentesis. CVS has the benefit of earlier diagnosis, but there is an increased risk of confined placental mosaicism which typically requires additional amniocentesis to clarify the foetal karyotype[30]. If there is an increased likelihood of a false positive result, amniocentesis may be the preferred option to minimize the mosaic potential. Further assisting decisions regarding prenatal testing is the increasing body of evidence that procedure-related miscarriage risk is lower than previously reported, with no significant difference to a control population not undertaking invasive procedures, and no significant difference between CVS and amniocentesis[31,32,33]. The possibility of a differential diagnosis such as Noonan syndrome is only introduced when there are structural abnormalities and PND has shown the NIPT result to be a false positive. This occurred in only two (1%) of our cases and further highlights the importance of 11-14-week ultrasound which may indicate other chromosomal or congenital abnormalities not assessed by NIPT[34].

The cfDNA test sample size is a strength of this study, providing one of the largest datasets of high probability MX results in the literature. However, the low prevalence of this condition still resulted in a modest sample size for exploratory regression analysis. This, together with the large number of potential explanatory variables and an important pattern of data missingness, meant that complete case data for all variables was only available in 24 cases (25%). As such, unbiased imputation was not possible and therefore multivariable model analysis could not be reliably undertaken[35]. Collaboration with other large screening units in future studies may allow multivariable analysis of the most important of these predictor variables in a larger and more complete data set. The differences between practices may limit the external validity of our findings and should be considered in future research.

Conclusion

There is limited literature on the prediction of false positive MX NIPT results and this study provides useful additional exploratory information. Ultrasound identified MX features and placental biomarkers are the primary factors of clinical utility. Predictive trends were identified in maternal age, BMI and ethnicity, however these factors cannot explain why MX carries such a low PPV compared to other aneuploidies assessed by NIPT. Whilst NIPT is an important advancement in the screening for common chromosome aneuploidies in pregnancy, assessment of ultrasound findings and placental serology in the first trimester remains important for appropriate post-test counselling and should continue to be a part of first trimester screening even in the setting of NIPT as a first-tier screening test.

Abstract

Objective: The accuracy of cell-free DNA aneuploidy screening varies by the chromosome assessed. The positive predictive value is consistently low for monosomy X (MX), at less than 30%. This study aims to investigate maternal age and other possible predictors of false positive MX screening results in order to guide pre-test and post-test counselling.

Methods: 52,499 NIPT samples were tested over 69 months, across 3 specialist obstetric services. Outcome data was available for 96 out of 107 cases high risk for MX. Cytogenetic outcomes were compared to clinical and demographic data to look for trends which may indicate higher likelihood of a false positive NIPT result.

Results: The likelihood of a false positive MX result significantly increased with the absence of ultrasound features suggestive of MX and with lower PAPP-A levels. Non-significant trends towards false positive results were identified with increased maternal age, increased body mass index and Caucasian ethnicity.

Conclusion: Maternal age is not a reliable predictor of a false positive result. Assessment of ultrasound findings and placental serology in the first trimester is important for appropriate post-test counselling and should continue to be a part of screening even when NIPT is used as a first-tier screening test.

Increasing maternal age is not a significant cause of false positive results for Monosomy X in non-invasive prenatal testing

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Data availability: 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.

Figure 1: Lowess curves relating A) maternal age, B) body mass index, C) PAPP-A and D) nuchal translucency measurement to the percentage chance of a false positive result.

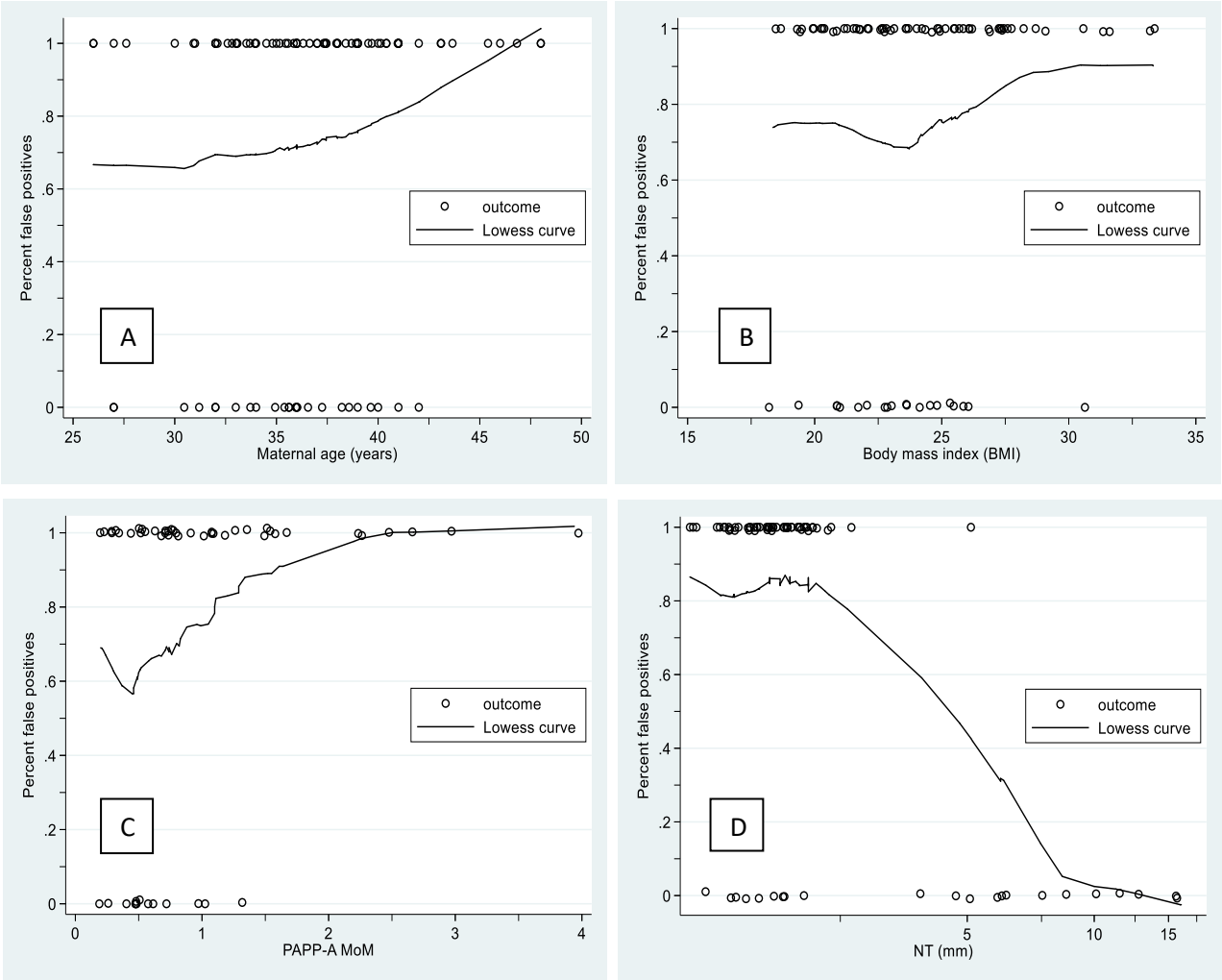
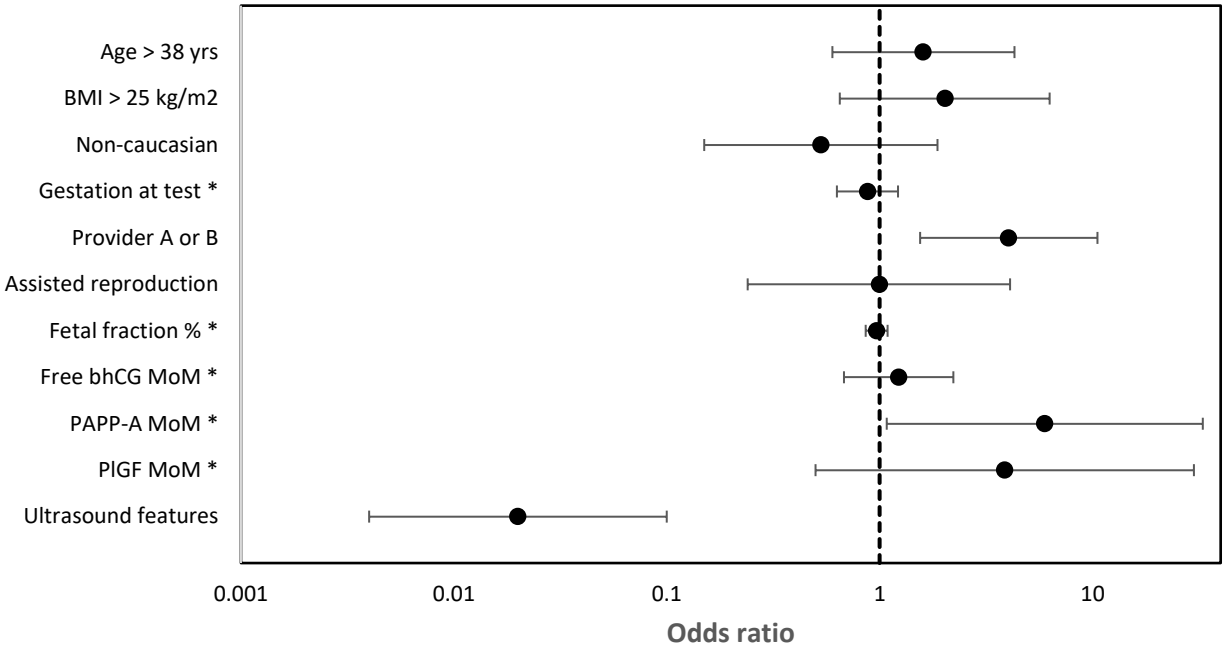


Figure 2: Effect size (odds ratio and 95% confidence intervals) of predictor variables and probability of a false positive result



* Effect size determined as a continuous variable as no clear dichotomisation point was obtained

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Table 1: Baseline characteristics of the high-risk monosomy X population.

Demographic variables	N*	n	(%)
Maternal age (years) – mean (SD)	96	36.4	(4.6)
Body mass index (BMI) (kg/m^2) – mean (SD)	82	24.2	(3.5)
BMI categories	82		
Underweight (< 18.0)		2	(2)
Normal (18.0 – 24.9)		50	(61)
Overweight (25.0 – 29.9)		24	(29)
Obese (≥ 30.0)		6	(7)
Ethnicity	77		
Caucasian		64	(83)
East Asian		10	(13)
South Asian		3	(4)
Service provider	96		
A		23	(24)
B		42	(44)
C		22	(23)
D		9	(9)
Clinical variables			
Gestation at cfDNA test (weeks) – median (IQR)	96	11.0	(10.4, 11.7)
Fetal fraction (%) – mean (SD)	79	9.4	(4.2)
Placental serology (MoM)			
Free β hCG – mean (SD)	54	1.66	(1.12)
PAPP-A – mean (SD)	54	0.99	(0.77)
PIGF – mean (SD)	37	0.97	(0.41)
Method of conception	84		
Spontaneous		72	(86)
Assisted reproduction		12	(14)
Ultrasound features of monosomy X	90		
Positive features		16	(18)
No features		74	(82)

** Number with recorded information in the final cohort*

Table 2: Distribution of the possible explanatory variables on the likelihood of a false positive monosomy X cell-free DNA result.

Demographic variables	False Positive n (%)	True Positive n (%)
Maternal age (years)		
≤ 38	44 (71%)	18 (29%)
> 38	27 (79%)	7 (21%)
Body mass index		
<25 kg/m^2	37 (71%)	15 (29%)
≥25 kg/m^2	25 (83%)	5 (36%)
Ethnicity		
Caucasian	48 (75%)	16 (25%)
Non-Caucasian	8 (62%)	5 (38%)
Gestation at cfDNA test		
< 11.6 weeks	54 (76%)	17 (24%)
≥ 11.6 weeks	17 (68%)	8 (32%)
Service provider		
A and B	54 (83%)	11 (17%)
C and D	17 (55%)	14 (45%)
Clinical variables		
Conception method		
Spontaneous	54 (75%)	18 (25%)
ART	9 (75%)	3 (25%)
Fetal fraction		
< 10%	33 (80%)	8 (20%)
≥ 10%	26 (68%)	12 (32%)
Placental serology		
Free beta hCG		
< 1.57 MoM	22 (69%)	10 (31%)
≥ 1.57 MoM	18 (82%)	4 (18%)
PAPP-A		
< 0.99 MoM	22 (65%)	12 (35%)
≥ 0.99 MoM	18 (90%)	2 (10%)
PlGF		
< 0.92 MoM	12 (63%)	7 (37%)
≥ 0.92 MoM	16 (89%)	2 (11%)
Ultrasound features MX		
No features	65 (88%)	9 (12%)
Positive features	2 (1%)	14 (87%)