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Implications of failure to achieve a result from prenatal maternal serum cell-free DNA testing: a historical cohort study

Shortened Running Title: Implications of NIPT failure: a retrospective cohort study

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Abstract

Objective:

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To investigate the pregnancy outcomes in a cohort of women who failed to obtain a result in non-invasive prenatal testing (NIPT)

Design:

Historical cohort study

Setting:

A multicenter private practice in Sydney, Australia

Population:

Women who failed to obtain a result from NIPT (n=131)

Methods:

The maternal characteristics, antenatal investigations and pregnancy outcomes for these women were compared to those who obtained a result at the same practice and to the general Australian obstetric population.

Main Outcome Measures:

Antenatal investigations: PAPP-A, free beta hCG, PIGF, uterine artery pulsatility index (PI), mean arterial pressure (MAP)

Pregnancy outcomes: chromosomal abnormality, pre-eclampsia (PET), gestational diabetes (GDM), small for gestational age (SGA), preterm delivery

Results:

1.1% NIPT samples failed to return a result. This cohort was significantly older and had significantly increased weight compared to the general Australian obstetric population.

Pregnancy outcomes were available for 94% of the cohort. There were significantly higher rates of chromosomal aneuploidies (6.5% vs 0.2%, $p<0.0001$), pre-eclampsia (11% vs 1.5%, $p<0.0001$) and gestational diabetes (23% vs 7.5%, $p<0.0001$) compared to the general obstetric population. Rates of preterm delivery and SGA were elevated but did not reach significance.

Antenatal investigations demonstrated decreased PAPP-A MoM (0.75 vs 1.14, $p<0.0001$), decreased fbhCG (0.71 vs 1.01, $p<0.0001$) and increased uterine artery PI (1.79 vs 1.65, $p=0.02$).

Conclusion:

Women who fail to obtain a result from NIPT are at increased risk of adverse pregnancy outcomes, in particular chromosomal aneuploidy, gestational diabetes and pre-eclampsia.

Funding:

Nil

Keywords:

cell-free DNA, prenatal, NIPT failure, fetal fraction, adverse pregnancy outcome

Tweetable abstract:

Women who fail to obtain a result from cell-free DNA NIPT are at increased risk of adverse pregnancy outcomes

Introduction

Cell-free fetal DNA was first discovered in maternal plasma 1997 by Lo et al. and has since become the focus of new technologies for prenatal screening(1).

Non-invasive prenatal testing (NIPT) involves the analysis of cell free DNA fragments in maternal serum samples. During pregnancy, cell free DNA consists of maternal fragments, primarily from haemopoietic cells, and fetal fragments, released from trophoblasts during apoptosis(1). In the original technology, these DNA fragments were all sequenced, their chromosomal origins identified using the maternal genome as a reference and processed by counting algorithms to identify molecular imbalances(2). The development of directed amplification and sequencing has allowed for a more targeted process(3). Some laboratories are now returning to more complete whole genome sequencing which can identify more complex genetic disorders including subchromosomal genetic disorders such as microdeletion syndromes(2, 4, 5).

In Australia, the current application of NIPT is in screening during the first trimester for the common fetal chromosome abnormalities (trisomies 21, 18, 13 and the sex chromosome aneuploidies), often as an adjunct to combined first trimester screening(5). The clinical validity of NIPT as a screening tool has been demonstrated by multiple studies with reported sensitivities of 99.7%, 98.2% and 99.0% for trisomies 21, 18 and 13 respectively, for a combined false positive rate of 0.13%(6-8).

Approximately 2-3% NIPT return an inconclusive result(9, 10). There are a number of reasons for failing to achieve a result(8). These include logistic failures due to errors in specimen collection, transport and storage or with laboratory processing as well as technical assay failure due to failed DNA extraction, amplification or sequencing. This latter group includes those with genomic disturbance such as maternal, placental or fetal mosaicism, malignancy(11) or in other (non-reported, normalizing) chromosomal abnormalities. The most common cause, however, is insufficient fetal cell-free DNA fraction(8).

Fetal fraction varies between individuals and throughout the pregnancy. There are two well-established influences on fetal fraction: gestational age being positively correlated while maternal weight has an inverse relationship (12). The influence of other factors such as smoking, ethnicity and chromosomal aneuploidy is less clear (10, 13). However, there are women with low fetal fraction whose results cannot be easily accounted for by these factors.

As the use of NIPT becomes more widespread, it is increasingly important to determine any clinical implications of NIPT failure. This study aims to explore the relationship between NIPT failure and adverse pregnancy outcome.

Materials and methods

Study Design

Historical cohort study.

Participants and Setting

Pregnant women who undertook NIPT at a private specialist, multi-site prenatal screening service in Sydney, Australia between June 2013 and March 2016 and failed to achieve a result were included in the study. No patients were excluded from the study.

Procedure

Prior to the NIPT, all patients underwent a sonographic assessment to confirm fetal number and viability and to accurately determine gestational age. Pre-test counseling regarding the scope and limitations of NIPT was provided by genetic counselors or fetal medicine specialists. Routine venepuncture was performed to collect the sample for NIPT.

Initially, samples were transported to Ariosa Diagnostics, California, USA for analysis by Harmony™. Harmony™ reported a test failure where samples had less than 4% fetal fraction or when assay disturbances or laboratory processing errors occurred. Availability of testing in Australia led to a change in NIPT provider during the study period with samples being sent to Genea, Sydney for analysis by GeneSyte®. Genomic disturbances, assay failure and technical causes could result in an inconclusive result with this platform, but fetal fraction was not measured in the study period, relying instead on sequencing depth and increased read numbers to maintain screening accuracy(14).

Women who failed to achieve an NIPT result were given the option of sample recollection for NIPT at no additional cost using either the original testing platform or an alternative NIPT provider, or alternatively proceeding with another prenatal screening or testing pathway. Those with repeat NIPT failures were referred to a fetal medicine specialist for counseling and given the option of definitive prenatal testing by chorionic villus sampling (CVS) or amniocentesis.

Data collection

Maternal characteristics (age, weight and height) and the results of any antenatal investigations comprising the first trimester screening, screening for pre-eclampsia and the ultrasound examination were collated. These included the placental biomarkers (free beta human chorionic gonadotrophin [fbhCG], pregnancy-associated plasma protein-A [PAPP-A] and placental growth factor [PIGF]) and maternal biophysical information (mean arterial pressure and mean uterine artery pulsatility index) (15). Outcome information including pregnancy complications, delivery mode, neonatal characteristics and progress was self-reported by the study participants via telephone interview conducted after the conclusion of the pregnancy.

The following definitions were used:

Preterm: delivery prior to 37 weeks gestation

Small for gestational age: weight less than 10th percentile adjusted for gestational age

Pre-eclampsia: Hypertension and coexistence of at least one of proteinuria, uteroplacental dysfunction, maternal organ dysfunction (renal insufficiency, liver involvement, neurological complications)

Gestational Diabetes Mellitus: Any of fasting plasma glucose 5.1-6.9mmol/L, 1hr post 75g oral glucose load >10mmol/L, 2hr post 75g oral glucose load 8.5-11mmol/L

Statistical Analysis

Due to data availability, there were 2 cohorts used as comparative controls; the general Australian obstetric population using data published by the Australian government (16-19) and an NIPT success cohort from the same private practice (7, 20). Analysis was conducted on the entire cohort of NIPT failures.

The normality of maternal age and BMI in the NIPT failure cohort was confirmed using Shapiro-Wilks test. This was compared to the general Australian obstetric population and the NIPT success cohort using student t-tests. The results of antenatal investigation were expressed as multiples of the median (MoM) and were compared to the NIPT success cohort using Mann-

Whitney tests as normality of these could not be confirmed. The incidence of pregnancy outcomes was compared to the general Australian obstetric population using binomial tests. Separate subgroup analysis was also performed on those patients with low fetal fraction and repeat NIPT failures. Statistical analysis was performed using R version 3.3.2(21).

Ethical Clearance

Ethical approval was provided by GENE Human Research Ethics Committee (10 June 2015, GEC0022).

Results

Characteristics of study population:

A total of 12033 women underwent NIPT at SUFW during the study period, with 6375 (53%) undertaking Harmony™ tests and 5658 (47%) undertaking GeneSyte® tests. There were 131 participants who received an inconclusive result (1.1%) of whom 119 (90.8%) were conducted by Harmony™ and 12 (9.2%) by GeneSyte®. The Harmony™ inconclusive test result percentage at 1.9% (119/6375) was significantly higher than that for GeneSyte® (0.2%, 12/5658; $p < 0.0001$; binomial test). Low fetal fraction was reported as cause of NIPT failure in 59 cases (45%) of the cohort and technical assay failure or logistical issues in 59 women (45%). In 13 cases (10%), the reason for failure was not reported.

Women who failed to obtain a result from NIPT had a higher mean BMI when compared to those who received a result (28.5 v 23.3 kg/m²; $p < 0.0001$) as well as to the general Australian obstetric population (28.5 v 26.0 kg/m²; $p = 0.0001$). They were older than the general Australian obstetric population (35 v 30 years; $p < 0.0001$) but younger than those with test success at the same clinical practice (35 v 36.2 ; $p = 0.001$). The 59 women who failed to achieve a result due to low fetal fraction had significantly increased BMI (30.8 vs 28.5 , $p = 0.04$) compared to the remainder of the cohort. No other significant demographic or pregnancy outcome metrics was found. There was no difference in gestational age at testing between those who failed to achieve a test result and those with test success (11.4 v 11.3 weeks; $p = 0.6$).

On review of placental function markers, women with test failure had significantly lower levels of PAPP-A and fbhCG as well as significantly higher uterine artery PI than those with test success, but there was no significant difference in PIGF. The comparisons are detailed in Table 1. There

were no differences in these investigations between those with test failure due to low fetal fraction and those whose tests failed due to technical laboratory issues.

With regards to pregnancy outcomes in the test failure cohort, 85 women (64.9%) had first trimester pre-eclampsia screening. Of those, 13 (15.2%) had a risk greater than 1 in 50, which was higher than found in routine screening of a comparable Sydney obstetric population, but did not reach significance (15.2% vs 9.9%, $p=0.1$)(22). Of the high pre-eclampsia (PET) risk group, 6 women (46%) developed PET and 3 delivered before 34 weeks gestation.

Screening pathway after NIPT test failure.

Of these 131 women who failed to obtain a result, 75 underwent a subsequent sample collection for NIPT either with GeneSyte®, Harmony™, Verifi™ or Panorama™, while the remainder pursued alternative screening pathways as detailed in Figure 1. Of the 75 who underwent a repeat NIPT, 13 (17.3% of all repeat samples; 28.3% of repeat Harmony™ samples) again received an inconclusive result.

Pregnancy outcomes:

Despite extensive efforts to contact the patients by telephone and post, pregnancy outcomes for 8 women (6%) are unknown. There was no significant difference in age, BMI or gestation at NIPT between these 8 women and the 123 women for whom outcomes are known. In the final cohort of 123 women, there were 110 livebirths and 13 cases with adverse pregnancy outcome (10.1% of those with known outcomes and 10.0% of the total cohort). The adverse outcomes included 7 terminations of pregnancy (including 6 cases of chromosome abnormality), 5 miscarriages (2 with chromosomal abnormalities) and 1 stillbirth. Pregnancy outcomes and relevant clinical information are detailed in Table 2.

There were 8 cases of chromosomal aneuploidy in these 123 pregnancies (6.5% of those with known outcomes and 6.1% of the entire cohort), which is significantly higher than the 0.2% rate in the general obstetric population ($p<0.0001$). The majority of these involved uncommon aneuploidies and three (38%) of these had low fetal fraction as the reported reason for NIPT failure.

Of the 110 livebirths, the incidence of pregnancy complications such as PET and GDM was significantly higher than the general obstetric population, whilst there was no significant

difference in rates of SGA or preterm delivery (Table 3). Of the 13 women who repeatedly failed to receive a result from NIPT, there was one case of triploidy resulting in miscarriage. No other chromosomal abnormalities were found in this group. Of the remaining 12 women, the birth outcome is known in 11 cases. Of these, six (55%) were complicated by GDM, one by SGA and one by PET.

There was no significant difference in the incidences of these adverse outcomes between the low fetal fraction group and the group that failed due to technical reasons.

Discussion

Main Findings:

In this study, we observed that the cohort of women who fail to receive a result from NIPT experience a significantly increased number of adverse outcomes including late miscarriage, chromosomal abnormality, pre-eclampsia and gestational diabetes.

The finding of uncommon chromosomal aneuploidies such as triploidy, trisomy 9 and trisomy 10 in the study cohort reflect an association with smaller placentae which is likely to be responsible for the abnormal cell free fetal DNA (cffDNA) levels and subsequent failure to obtain a result from NIPT(3). On the other hand, sex chromosome aneuploidy and trisomy 21 were also identified in this cohort, and these are considered unlikely to create abnormal placental volume and on the contrary, enhanced cell turnover is generally considered to create excess cffDNA in trisomy 21(23, 24).

With regards to antenatal investigations, PAPP-A and fbhCG were significantly lower in the NIPT failure cohort. Moreover, free bhCG was lower in the group failing due to low fetal fraction compared to those with failures for other reasons. This could be a reflection of poorer placental function and/or decreased placental volume. The increased uterine artery mean PI measurements and decreased PAPP-A are both associated with an increased risk of PET which correlates with the increased incidence of PET found in our study cohort. PET is theorized to develop as a result of impaired invasion by the trophoblasts during early gestation leading to abnormal placentation(25). It is feasible that this same pathological process might then cause abnormal apoptosis of these trophoblasts, resulting in abnormal amounts of cffDNA being released into the maternal blood that is required for analysis in NIPT(26).

The study cohort had a higher BMI than the general Australian obstetric population. The only significant difference demonstrated between those women who had insufficient fetal fraction and those who did not receive a result from NIPT for other reasons was an increased BMI, which suggests a dilutional effect and increased maternal adipocyte turnover as the principal causes(12). The increased BMI observed in our cohort may also act as a confounding factor when considering the apparent significant increase in rates of GDM and PET(27). Callway reported a correlation between BMI and both GDM and PET (unadjusted OR for BMI:25-29.9 of 1.78 and 1.74 for GDM and PET respectively)(28).

It was difficult to draw any conclusions regarding those who repeatedly failed to obtain a result due to the small numbers in this particular group.

The overall NIPT failure rate during the study period of 1.1% compares favorably with a recent systematic review of 11 studies reporting failure rates ranging from 0-4.9%(29). Since the analysis in NIPT is performed on all cell free DNA in the sample, a minimum fetal fraction (ranging from 2-4% total cell free DNA) is required in order for the test to have adequate statistical power. However, some commercial laboratories assert that reliable results from NIPT are possible without measuring the amount of fetal fraction. Takoudes et al. reported in 2015 on a sample challenge which resulted in non-pregnant women receiving a result from NIPT at some laboratories(30). In their theoretical study, Wright et al. found the detection rate of a method combining NIPT with combined first trimester screening test to increase from 62% to 100% as fetal fraction increases from 4% to $\geq 9\%$ (31).

Our study provides further evidence that increased BMI is correlated with decreased fetal fraction as has been established in previous studies(13, 23, 26) . Furthermore, other studies including that by Ashoor et al. found that fetal fraction also varied depending on ethnicity although this was not explored in our study(13). The reported reasons for failure in our study are distributed in a similar fashion to those demonstrated previously with both Benn and Norton reporting that around half of the 'no results' in their studies were attributable to low fetal fraction(10, 29).

Fetal fraction is affected by chromosomal abnormalities, with trisomy 21 having increased fetal fraction whilst trisomies 13, 18, monosomy X and triploidy may be associated with lower fetal fraction(3, 23, 24). We found an increased rate of chromosomal abnormalities (6.5% of the

entire cohort) in those who failed to receive a result from NIPT, concurring with previous studies. Norton in his study reported a significantly elevated aneuploidy rate (2.7%) compared to their overall cohort prevalence (0.4%) (10) as did Pergament et al, in their study in high risk patients (23% vs 11%)(32). This is compared to the general obstetric population incidence of 0.2%(19). As found in the current study, the chromosome abnormalities in these 'no result' cohorts tended to be the rarer aneuploidies, most often associated with small volume placentae. Gil et al. found in their retrospective study that pregnancies affected by trisomies and sex chromosome aneuploidies had significantly increased rates of NIPT failure (6.9% and 17.2% respectively)(33).

While we found an association between NIPT failure and PET, previous studies have focused specifically on fetal fraction and PET. The literature is inconclusive about this with some studies finding an elevation in fetal fraction before disease onset (8, 34-36) while others report no significant difference between pre-eclamptic and normal cohorts (37, 38). It is worth noting that the association with PET in our study was present in both our overall cohort and the low fetal fraction cohort, suggesting that there may be pathological processes involved, leading to not only decreased quantity but other abnormalities in the cfDNA. However, the increased age and BMI in our cohort may account for some of this increase(27, 39). This study found no significant increase in SGA or preterm birth and, again, the literature is mixed regarding the association of fetal fraction at 10-14 weeks and these conditions(35, 40-43).

Strengths:

The strengths of this study are the large number of NIPT tests conducted by a dedicated screening practice with access to skilled and appropriately trained medical and allied health personnel. There were sufficient test failures to provide robust comparative analysis.

Limitations:

Being conducted entirely in private practice, the cohort in this study may not be representative of the whole obstetric community. Moreover, as a private tertiary referral screening centre, decisions regarding patient management are made by the referring clinicians, rather than the screening practice specialists, which accounts for the variability in antenatal investigations performed. The retrospective nature of this cohort study means that there was limited control over data collection resulting in an incomplete dataset: there are 8 women (6%) for whom pregnancy outcomes are unknown despite repeated attempts at follow-up. This study is also

susceptible to recall bias as pregnancy outcomes were self-reported. Using different testing platforms in this study may also lead to lack of standardization within the failure cohort.

Interpretation:

This study demonstrated that the group of women who fail to receive a result from NIPT are at increased risk for adverse pregnancy outcomes including chromosome abnormalities and pregnancy complications such as pre-eclampsia and gestational diabetes. Moreover, the failure rate of NIPT indicates that there is still an important role for combined first trimester ultrasound and biochemical screening and for conventional invasive prenatal testing (CVS, amniocentesis) which rarely fails to report a result and also tests for a larger number of chromosomal and genetic abnormalities

Conclusion:

Failure to achieve a result from NIPT is associated with a higher risk for adverse pregnancy outcome. These women require careful counseling, possible definitive prenatal testing and more stringent antenatal monitoring. A potential future study in this cohort of women may investigate the placenta-maternal immunological interface through exosome testing to provide additional insight into abnormal placental function.

Disclosure of Interests:

The authors declare no conflict of interest. The ICMJE disclosure forms are available as online supporting information.

Contribution to authorship:

The maternal characteristics data and antenatal results were collated by RS and MS. RS, MS, NC and AM gathered pregnancy outcome data. NC analysed the data and prepared the tables and figures with revision by AM and FC. NC wrote the manuscript with revision by FC and AM.

Details of ethics approval:

The study protocol received ethical approval from Geneva Human Research Ethics Committee (10 June 2015, GEC0022).

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Antenatal Investigation	NIPT Failure Cohort Median (sample size)	NIPT Success Cohort Median (sample size)	P value (Mann-Whitney Test)
PAPP-A MoM	0.75 (99)	1.14 (2469)	<0.0001
PIGF MoM	0.93 (58)	1 (2252)	0.07
Free bhCG (MoM)	0.71 (100)	1.01 (12515)	<0.0001
Uterine Artery PI	1.79 (86)	1.65 (2959)	0.02

Table 1: Placental functions markers in test failure cohort compared to an NIPT cohort with test success from the same practice (fbhCG – free beta human chorionic gonadotrophin; PAPP-A - pregnancy associated placental protein-A; PIGF – placental growth factor. PI – pulsatility index; MoM – multiple of the median).

Adverse Outcome (n)	Gestation and Condition	NIPT Result	Relevant abnormal findings
Miscarriage (5)	14 weeks (triploidy)	NIPT failure due to insufficient fetal cfDNA Repeat NIPT failure with same provider	PAPP-A 0.05
	13 weeks (trisomy 9)	NIPT failure due to insufficient fetal cfDNA Repeat NIPT with same provider was successful	hCG 0.18; PAPP-A 0.4; PLGF 0.42
	19 weeks	No reason given for NIPT failure	Incompetent cervix
	13 weeks	NIPT failure due to insufficient fetal cfDNA	Nil
	16 weeks	NIPT failure due to unusually high variance in cfDNA counts Repeat NIPT with same provider was successful	Incompetent cervix. FbhCG 1.78, PAPP-A 1.45, PIGF 0.7; risk of T21: 1/244
Termination of Pregnancy (7)	Mosaic Trisomy 10	NIPT failure due to chromosomal anomalies	FbhCG 3.23, PAPP-A 0.11, UA PI 1.27. Enlarged cerebral ventricles, posterior fossa defect, congenital diaphragmatic hernia
	Trisomy 9	NIPT failure due to unusually high variance in cfDNA counts Repeat NIPT with same provider was successful	Cardiac defect, micrognathia, sandal gap and echogenic kidneys
	Trisomy 13	NIPT failure due to insufficient fetal cfDNA	NT 4.4, fbhCG 0.23, PAPP-A 0.43, PIGF 0.66, UA1.54. Central umbilical cord cyst, posterior fossa cyst
	XXY	NIPT failure due to chromosomal anomalies	Nil
		NIPT failure due to insufficient fetal cfDNA	

	Triploidy		fbhCG 0.13, PAPP-A 0.07, UA PI 1.49. Early SGA,
	XXX	NIPT failure due to chromosomal anomalies	Nil
	Euploid	NIPT failure due to insufficient fetal cfDNA Repeat NIPT with different provider was successful	PPROM at 19w 2d. Persistent severe oligohydramnios, TOP at 22w.
Stillbirth (1)	25 weeks	NIPT failure due to unusually high variance in cfDNA counts Repeat NIPT with different provider was successful	High risk for T21 (1/25) and high early onset PET risk (1/51). Severe SGA. No genetic testing.

Table 2. Adverse pregnancy outcomes in women receiving an inconclusive NIPT result. SGA – small for gestational age. PPRM – preterm, pre-labour rupture of membranes. TOP – termination of pregnancy. NT – nuchal translucency

Clinical outcome in pregnancies resulting in livebirth	Australian Population Incidence %	Overall Study Cohort Incidence %	P value (binomial test)
GDM	7.5	20 (25/123)	<0.0001
PET	1.5	9.8 (12/123)	<0.0001
SGA	10	15 (16/106)	0.1
Preterm	8.5	11 (14/123)	0.3
Chromosomal Aneuploidies	0.2	6.5 (8/123)	<0.0001

Table 3. Pregnancy complication rate in women with NIPT failure and known outcomes. GDM – gestational diabetes mellitus. PET – pre-eclampsia. SGA – small for gestational age.

Figure 1: Screening pathway following NIPT testing including with pregnancy outcome where known.

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