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Clinical experience with non-invasive prenatal screening for single-gene disorders (NIPT-SGD)

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Contribution:

What are the novel findings of this work?

A total of 2,208 women received a non-invasive prenatal test result for a panel targeting 25 clinically significant single gene disorders. High test positive rates were demonstrated for women with ultrasound abnormalities or with a family history of one of these disorders.

What are the clinical implications of this work?

The results demonstrate the potential for early detection for these disorders through cell-free DNA analysis.

Abstract

Objectives

To assess the performance of a non-invasive screening test for a panel of dominant disorders with a combined population incidence of 1 in 600. The panel included 30 genes covering 25 disorders including Noonan spectrum disorders (NSDs), skeletal disorders, craniosynostosis syndromes, Cornelia de Lange syndrome (CdLS), Alagille syndrome, tuberous sclerosis, epileptic encephalopathy, SYNGAP1 related intellectual disability, CHARGE syndrome, Sotos syndrome and Rett syndrome.

Methods

Cell-free fetal DNA isolated from maternal plasma samples accessioned from April 14, 2017, to November 27, 2019, was analyzed by next generation sequencing, targeting 30 genes, to look for pathogenic or likely pathogenic variants implicated in 25 dominant disease conditions. Testing was offered for women with singleton pregnancies at 9 weeks gestational age or later, with testing on maternal and paternal genomic DNA to assist in interpretation. A minimum 4.5% fetal fraction was required for test interpretation. Variants identified in the mother were deemed inconclusive with respect to fetal carrier status. Confirmatory prenatal or postnatal diagnostic testing was recommended for all screen-positive patients and follow-up information was requested.

Results

Results were available for 2,208 women, 125 (5.7%) of which were positive. Elevated test-positive rates were found for referrals with a family history of a disorder on the panel, 20/132 (15.2%); a primary indication of fetal long bone abnormality 60/178 (33.7%); fetal craniofacial abnormalities, 6/21 (28.6%); fetal lymphatic abnormalities 20/150 (13.3%) and major fetal cardiac defects 4/31 (12.9%). For paternal age ≥40 years as a sole risk factor, the test positive rate was 2/912 (0.2%). Of the 125 positive cases, follow-up information was available for 65 (52%) with none classified as false-positive. No false-negative cases were identified.

Conclusions

Non-invasive prenatal testing can assist in the early detection of a set of single gene disorders, particularly when either abnormal ultrasound findings or a family history is present. Additional clinical studies are needed. NIPT-SGD offers a safe and early prenatal screening option.

Introduction

The presence of cell-free fetal DNA (cffDNA) in maternal plasma was described by Lo et al., in 1997.[1] However, only in the last decade has molecular genetic technology advanced sufficiently to allow clinical implementation of cffDNA based non-invasive prenatal testing (NIPT), which is now commonly used in the detection of fetal chromosome abnormalities.[2-5] Originally targeting trisomies 21, 18 and 13, NIPT has expanded in scope to include sex chromosome aneuploidies,[4,6] and a select group of microdeletions.[7]

Single gene disorders are present in approximately 1% of births.[8] Non-invasive prenatal testing for single gene disorders (NIPT-SGD) is feasible for a broad range of monogenic disorders, and is most straightforward when applied to the detection of dominant conditions with a high de novo rate or for paternally inherited dominant gene variants.[9, 10] A currently available panel for NIPT-SGD screens for 25 conditions that result from disease causing variants across 30 genes (Supplemental File 1), which have a combined incidence of 1/600 (0.17%).[11] The conditions include Noonan spectrum disorders (NSDs), skeletal disorders, craniosynostosis syndromes, Cornelia de Lange syndrome (CdLS), Alagille syndrome, tuberous sclerosis, epileptic encephalopathy, *SYNGAP1*-related intellectual disability, CHARGE syndrome, Sotos syndrome and Rett syndrome. Although the testing can be considered as fully diagnostic for the conditions detected (non-invasive prenatal diagnosis, NIPD),[8] we will refer to this testing as “screening”.

The clinical features associated with the disorders screened can vary widely, and therefore present challenges in establishing a diagnosis. Some of these conditions will not be recognized by first/second/third trimester ultrasound examination but can be diagnosed after birth with physical or cognitive disability and may require surgical/medical intervention or affect quality of life. In addition, clinical features in the prenatal setting do not always correlate with the postnatal presentation. Supplemental File 1 summarizes the clinical features associated with this set of disorders.

A previously published initial validation of this 30 gene prenatal screening panel for monogenic disorders involved the demonstration of assay performance on 422 maternal plasma samples, 32 of which had genetic variants targeted by the panel.[11] In the current study, we present our clinical experience applying NIPT-SGD to a larger cohort of women who received this test. We further analyze the screen-positive rates based on the clinical indications for testing.

Methods

This study retrospectively analyzed a cohort of NIPT-SGD test requests received between April 14, 2017, and November 27, 2019. Testing was made available as a clinical service to all types of obstetric providers for singleton pregnancies at 9 weeks gestational age and later, using the brand name of Vistara. Clinicians had access to educational brochures, Natera's provider educational modules, publications, and other on-line resources. Patients were informed by their clinical providers about the benefits and hazards and of the testing and patients provided consent for testing. In addition to resources available at some of the practices, pre- and post- test genetic information sessions were available through Natera's in-house staff of genetic counselors. Information requested by the laboratory at the time of referral included the presence of abnormal ultrasound findings, family history for a single gene disorder, and age of both parents. If the pregnant woman was known to be affected with one of the conditions on the panel, that variant was excluded from test interpretation since the plasma is a mixture of maternal and fetal DNA and differentiating them is problematic. Fetal risk assessment was still available for other variants within that gene and other conditions for which the pregnant woman was not affected. A maternal age ≥35 years and a paternal age ≥40 years were considered to be "advanced" maternal and paternal age, respectively.

All testing was performed and interpreted in a College of American Pathologists and Clinical Laboratory Improvement Act approved reference laboratory (Baylor Genetics, Houston, Texas). Maternal venous blood (collected in BCT Streck tubes), paternal venous blood (EDTA tubes,) or paternal saliva (Oragene tubes) were collected to screen singleton pregnancies at ≥9 weeks gestation. Testing was performed on samples that met quality metrics, which included a 4.5% minimum fetal fraction, and a minimum next generation sequencing (NGS) coverage of at least 97% of the target regions. NIPT-SGD involved sequencing of coding exons, and 10bp exon/intron boundaries in DNA extracted from maternal plasma, maternal genomic DNA, and paternal genomic DNA (trio testing). This process included library construction, target gene enrichment, and NGS. A complete description of the assay development and analytical validation was previously published by Zhang et al.[11] Fetal fraction was determined by using a SNP-based approach by measuring the paternal allele contribution in the maternal plasma.[5,12] Paternal DNA was also used for quality control and for variant classification purposes.

NGS data from each trio was evaluated in aggregate, and variants were curated as pathogenic or likely pathogenic based on American College of Medical Genetics (ACMG) guidelines.[13] Before reporting, positive variants were confirmed by a secondary amplicon-based NGS assay using

deeper sequencing (>10,000X). Variants of unknown significance were not reported. Confirmatory prenatal or postnatal diagnostic testing was recommended for all screen-positive patients.

For the purposes of assessing frequencies of single gene disorders in various subgroups of referrals, and, recognizing that some cases had multiple reasons for referral, we classified each case according to the expected largest risk factor. The order of this classification, from high to low risk, was: (1) family history (parent affected, carrier or previous affected relative for a disorder on the screening panel); (2) presence of an abnormality of long-bones (primarily shortened), cranial/facial abnormality; (3) cardiac defect (all heart defects except echogenic focus); (4) lymphatic system defect (hydrops, pleural effusion, cystic hygroma or increased nuchal translucency); (5) other or unspecified ultrasound abnormality; (6) advanced paternal age; (7) advanced maternal age; (8) unspecified reason for referral. Advanced paternal age was the assumed indication if the paternal age was ≥40 years and no other indication was listed.

Collection of the data in this study was conducted as part of a quality assurance program, and the data was de-identified before analysis. This study was determined to be exempt from Investigational Review Board (IRB) approval (E&I IRB: 20099-01). In addition to collecting follow-up data provided voluntarily by clinics, we actively contacted clinics with positive NIPT-SGD results to evaluate the clinical validity of the testing. The data set present in this manuscript includes the previously reported cases,[11] and one which was published as an individual case report.[14]

Results

During the period from April 8, 2017, to November 27, 2019, a total of 2,416 cases were accessioned for the NIPT-SGD 30 gene panel. 132 (5.5%) tests were cancelled upon accessioning due to lack of eligibility/damaged specimen, and 76 (3.1%) did not pass quality metrics after processing. Samples that failed quality metrics were because of poor DNA quality, low fetal fraction, low fetal DNA yield after amplification or misidentified parentage. Figure 1 summarizes the cases in the cohort. Table 1 summarizes the indications for testing and the demographics for the referrals for NIPT-SGD and a result.

For all tests, 56.3% (1360/2416) were referred by specialty Maternal Fetal Medicine (MFM) practices with an available geneticist or genetic counselor, 30.6% (736/ 2416) were referred by general OBGYN practices and in 13.1% (317/2416) the referral group specialty was unknown. For cases with abnormal ultrasound findings, 74.7% (384/514) were referred by specialty MFM/genetics practices, 13.2% (68/514) were referred by general OBGYN groups and in 12.1% (62/514) the referral group specialty was unknown. For cases with positive family history, 51.5% (68/132) were referred by specialty MFM/genetics practices, 29.5% (39/132) were referred by general OBGYN groups and in 18.9% (25/132) practice type was unknown.

Of the 2,208 cases with a NIPT-SGD result, 125 (5.7%) were test-positive for a single gene disorder. Table 2 summarizes the number of affected pregnancies identified for each of the major groups of conditions included in the screening. Supplemental Table 1 provides the specific gene variants and additional clinical details for these positive test cases. Of the total number of variants identified, 104 were classified as pathogenic and 21 likely pathogenic. There were 25 variants that were considered novel (i.e., variants for which no published or database information existed to fully assess the phenotype, but based on the molecular change, could reasonably be inferred as being clinically consequential). These variants were considered likely pathogenic in accordance with ACMG guidelines and therefore included in the reported information.

Table 3 summarizes the breakdown of positive NIPT-SGD results by indication for testing. There were 132 cases tested because of a family history for one of the conditions on the panel. These cases included those in which the father was known to be affected, the couple had a previously affected child, or a more distant affected family member existed. In the 132 cases with a family history, positive results for the fetus were found in 20 (15.2%) cases; including 17 with the autosomal dominant condition known to be derived from the father, two in the mother (not known at time of referral), and one with a previous child. Additionally, in 12 cases, a parent was

diagnosed as affected because of a positive NIPT-SGD (10 maternal and 2 paternal discoveries). We obtained clinical confirmation via diagnostic testing in 50% (5/10) of the maternal cases discovered as a result of NIPT-SGD testing, and in 2 out of 10 cases the clinic confirmed clinical features suggestive of NSD in both the patient and a previous child. In both paternal cases discovered as a result of NIPT-SGD, the fetal diagnosis was confirmed by postnatal clinical exam.

There were 514 cases tested because of abnormal ultrasound findings (Table 3) of which 99 (19.3%) had NIPT-SGD positive results. Tests performed because of an abnormal ultrasound were referred significantly later in pregnancy (mean 22.0 weeks gestational age) compared to those tests performed for other reasons (mean 14.1 weeks) ($p< 0.001$). (Tables 1 and 4). For those performed because of an abnormal ultrasound finding, the gestational age at testing was later for those with positive NIPT-SGD results (mean 24.3 weeks) compared to those with abnormal ultrasound findings and a negative NIPT-SGD result (mean 21.4 weeks) ($p<0.001$).

Among the 514 cases performed because of an abnormal ultrasound, the most common primary indication involved shortened or abnormal long bones (178/514, 34.6%). This group also showed the highest rate of positive tests (60/178, 33.7%). Of the 60 positive cases with this reason for referral, 22 were associated with osteogenesis imperfecta, 19 thanatophoric dysplasia, and 13 achondroplasia/hypochondroplasia. The less common conditions were NSD (2), CdLS (2), Apert (1) and Alagille syndrome (1). A high test-positive rate was also observed for referrals with cranial/facial (6/21, 28.6%) defects.

Referrals with lymphatic system abnormality (increased NT/cystic hygroma/pleural effusion) were also common, comprising 150 (29.2%) cases. Of the 20 positive cases in this group, 18 were variants associated with NSDs. There was also one case of CdLS, and one with osteogenesis imperfecta.

Of the 31 (6%) abnormal ultrasound finding referrals with a primary indication of cardiac abnormality, 4 had positive NIPT-SGD findings. None of the variants were associated with an NSD; the positive results were for tuberous sclerosis (3) and CdLS (1).

There were 912 tests with advanced paternal age (≥40 years, APA) and no other specific indication. The APA test positive rate was 2/912 (0.2%) (positive cases had paternal ages 55 and 61, respectively). For comparison, 650 tests were performed on cases where paternal age was <40 years and no other indication for testing was recorded. Of these, 4/657 (0.6%) were positive (paternal ages 28, 28, 33, 36 years). Thus, while this data provided no direct evidence for a

paternal age effect, advanced paternal age was a common feature of the referral population, with an average paternal age of 39.5 years (Table 1). Moreover, among the 99 positive cases that had abnormal ultrasound findings, 18 (18%) were also APA. These data were therefore suggestive of a paternal age effect, but the dataset was too small and heterogeneous for additional investigation into paternal age as a contributing factor.

We attempted to gather information on additional testing and pregnancy outcomes for all the 125 NIPT-SGD positive cases. In the 125 NIPT-SGD screen positive women, 7 (5.6%) had previously received an amniocentesis/CVS with a normal karyotype and/or normal microarray result. All of these 7 cases had abnormal sonographic abnormalities. In addition, 31 were known to have received a normal NIPT result. In 30 (24%) cases, there was no additional screening (NIPT, maternal serum screening) or diagnostic testing pursued prior to the NIPT-SGD test. For the rest of the positive cases, the information was not available from the clinic, or the case was lost to follow up. For NIPT-SGD negative cases, comprehensive information for related testing was not available. However, at least 840/ 1661 (50.6%) received NIPT aneuploidy screening. This rate was a minimal estimate because of the incomplete ascertainment.

In 20 (16%) positive cases, the specific autosomal dominant disorder was known to be segregated from the father (18) or mother (2), and therefore confirmatory studies were deemed non-essential. Of the other 105 positive cases, confirmatory study results were available for 52 (49.5%). 38 were confirmed by prenatal or postnatal diagnostic testing, 11 were independently confirmed by postnatal clinical exam, and 3 were confirmed by maternal blood studies. In one additional case, in which maternal mosaicism was detected by NIPT-SGD, the risk for each subsequent pregnancy was therefore up to 50%. In this particular case, the maternal variant was not detected by fetal diagnostic testing. The remaining cases either planned to pursue diagnostic testing postnatally or were lost to follow up. Zero-false positive results and zero false negative results were reported.

Among the screen positive cases, 18.4% (23/125) reported an adverse outcome, including 2 cases of intrauterine fetal demise (IUFD), 4 stillbirths, 13 neonatal deaths, and 4 pre-term deliveries. There were also 7 elective pregnancy terminations.

Care providers were queried whether the NIPT-SGD positive result precipitated pregnancy management changes. This included a change in delivery location (e.g., referral to a pregnancy high-risk center), change in delivery plan (e.g., early induction or C-section instead of vaginal delivery), referrals to additional specialists, or altered post-delivery plans (including ensuring

NICU availability or opting for palliative comfort care). Of 43 responses, 17/43 [39.5%] responded “No”, and 26/43 [60.5%] responded “Yes”.

Discussion

In this study, we provide a report of our clinical experience with NIPT-SGD, which focuses on a specific set of dominantly inherited or de novo gene variants in 30 genes. In this clinical cohort, enriched for pregnancies at high risk for these disorders, 5.7% (125/2,208) tested positive. The highest test-positive rates were observed for cases tested because of a family history (15.2%), fetal long bone abnormalities (33.7%), fetal skull/facial malformations (28.6%), fetal lymphatic abnormalities (13.3%) and fetal cardiac abnormalities (12.9%). In addition to identifying causal gene variants in fetuses with fetal abnormalities, we identified previously unidentified carrier parents. Among cases with confirmatory follow-up testing, no false-positive or false-negative results were found.

Our results extend a validation study by Zhang et al.[11] In that study, a series of 422 cases were evaluated and in the 147 cases with independent confirmatory studies, 20 were true-positive, 127 were true-negative and there were no false-positive or false-negative results. In another small series, Yan et al., evaluated the same assay in 13 cases with skeletal abnormalities or increased nuchal translucency.[15] Yan et al identified 8 fetuses with monogenic disorders and 2 affected mothers, with no false-positive results. With 2,208 reported results, our study represents the largest cohort of NIPT-SGD used in clinical practice.

The genes included in the panel are expected to detect over 80% of all Noonan syndrome cases (NSD) (Supplemental File 1). Therefore, this testing has an obvious application for pregnancies where this group of disorders is under consideration due to the presence of polyhydramnios, cystic hygroma, increased NT, fetal hydrops and cardiac anomalies.[16] NSD have a combined incidence of 1/1000-1/2500.[17] They represent the second most common genetic cause of congenital heart defect,[18] with affected individuals presenting with multisystemic abnormalities associated with high both morbidity and mortality.[16] Prenatal diagnosis is advantageous because benefits from altered delivery management and neonatal medical interventions exist. Among the tests performed because of ultrasound evidence for lymphatic abnormalities, we found positive results for genes other than those typically associated with NSD. Likewise, within cases with ultrasound evidence for skeletal abnormalities, we found positive results for genes other than those typically associated with osteogenesis imperfecta, thanatophoric dysplasia, and achondroplasia (Supplemental File 1). These results indicate clinical benefit for the use of a broad NIPT-SGD panel for screening patients suspected of having one of these disorders.

Although the observed positive rate was less striking with 6/1,562 (0.4%) in the positive cases without abnormal ultrasound findings or family history (Table 3), NIPT-SGD could be beneficial for apparently sonographically normal pregnancies. Many of the conditions on the NIPT-SGD panel are associated with absent to nonspecific prenatal ultrasound findings (Supplemental File 1). Moreover, NIPT-SGD can be offered in the first trimester (in conjunction with NIPT to screen for aneuploidy), at which time the associated fetal anatomic abnormalities are typically not visible. In this study, of all sonographically abnormal cases with variants, 30/99 (30.3%) were identified only after the abnormal ultrasound findings were seen in the third trimester, beyond the recommended window for chorionic villous sampling (CVS) or amniocentesis. A further 62/99 (62.6%) of cases were only referred following identification of second trimester ultrasound abnormalities with limited time for a full diagnostic work-up. NIPT-SGD could also be more appropriate for pregnancies from older fathers because of the association between de novo variants and paternal age,[19] although no direct evidence for the association with paternal age could be demonstrated in this study. Freidman estimated that the risk for de novo pathogenic single gene variants for fathers aged >40 years was at least 0.3-0.5%.[20] Our dataset was too small and too heterogeneous to allow us to explore the paternal age association in detail.

Limitations to our study need to be emphasized. There were no strict inclusion criteria for access to this testing and therefore the referral population was probably enriched for those patients who were perceived by their healthcare providers as being most likely to benefit. Therefore, the cases studied were not necessarily representative of all pregnancies. We do not know the full extent to which some patients may have received additional testing to rule out aneuploidy or copy number variants which will affect the proportion of cases with positive results. Similarly, some patients with a family history for one of the disorders could have received other testing or used NIPT-SGD to avoid an invasive test. Follow-up was not received in all test-positive cases we contacted and post-delivery studies were not performed in all negative cases; therefore, we cannot exclude false-positive or false-negative results. Although the initial performance data indicates that a de novo or paternally inherited genetic variant can be detected with a high degree of certainty, we urge caution in concluding that the testing is diagnostic. Therefore, we advocate confirmatory testing on samples derived from invasive procedures.

Given that NIPT-SGD can potentially help detect some affected pregnancies, the question of how NIPT-SGD could be integrated into prenatal care bears asking. Standard of care currently recommends that pregnant women with abnormal ultrasound findings be offered invasive diagnostic testing through CVS or amniocentesis, with analysis by chromosome microarray.[21] For

those cases without an explanatory chromosomal imbalance, panel based molecular analysis or exome or genome sequencing (ES) can be considered. NIPT-SGD cannot be expected to identify the full genome-wide range of variants potentially identifiable by ES. On the other hand, ES is expensive and often requires 2-4 weeks to obtain results. Our observations justify additional clinical studies to evaluate the optimal design of the gene panel, define target populations, and assess patient acceptability of NIPT-SGD in various clinical scenarios.

Wider availability of NIPT-SGD brings additional challenges. Similar to ES, the testing does not identify promoter and deep intronic variants, copy number variations, and structural rearrangements. Many of the conditions show variable penetrance or expressivity. NIPT-SGD is currently unsuitable in evaluating a fetus for maternal inherited variants. In some cases, the testing will identify an inherited variant not previously known to be present in a parent. Despite these issues, we found that in positive result cases where feedback was received, 60.5% of clinical providers confirmed that management changes were made as a result of the positive NIPT-SGD results. NIPT-SGD is in its earliest stages of development and considerable potential exists to expand its scope through the sequencing of more genes.

In summary, our study demonstrated the potential value of NIPT-SGD, notably when abnormal ultrasound findings are seen, or with a relevant family history. If correctly implemented with close counseling and monitoring, NIPT-SGD offers a safe and timely prenatal screening option.

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Conflict of Interest

P. Mohan, J. Lemoine C. Trotter, I. Rakova and P. Billings are employees of Natera, Inc. with stock and/or options to own stock in the company. S. Peacock declares ownership of Natera stock. C-Y. Kao, Y. Wang, F. Xia, and C. Eng report no potential competing interests. P.Benn is a paid consultant to Natera, Inc. and holds stock options.

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Figure 1. Flow diagram for the cases included in the study

Supplemental File 1. Description of disorders and expected performance of the NIPT-SGT panel.

Table 1. Indications for testing and the demographics for the NIPT-SGA referrals with a result.

Indications*	**Number of cases (%)**
Family history	132 (6.0)
Abnormal ultrasound	514 (23.3)
Advanced paternal age	912 (41.3)
Unspecified indication	650 (29.4)
TOTAL	2,208 (100)
Demographics, mean	**Mean (s.d.)**
Paternal age (n= 2,208)	39.5 (9.1)
Maternal age (n= 2,208)	34.6 (5.9)
Gestational age, all indications (n= 2,156)†	15.9 (6.3)

* Indications were classified according to a hierarchical prioritization (see methods).

†52 cases with incorrect gestational age (GA) were excluded. Mean gestational age for cases without ultrasound abnormalities 14.1 weeks and 22.1 weeks for cases with ultrasound abnormalities, see also Table 4

Table 2. Disorders identified through NIPT-SGD

Disorder group	Number of positive cases identified	% of all test-positive	% of all results
Skeletal (a)	73	58.4	3.3
Craniosynostosis (b)	10	8.0	0.45
Noonan (c)	29	23.2	1.3
Other syndromic disorders (d)	13	10.4	0.59
Total	125	100	5.7

(a) Includes osteogenesis imperfecta, thanatophoric dysplasia, achondroplasia, and hypochondroplasia

(b) Apert, Pfeiffer and Crouzon syndromes

(c) Noonan syndrome, Costello syndrome, cardiofaciocuteneous syndrome, and Noonan-like syndrome with loose anagen hair

(d) Tuberous sclerosis, Alagille syndrome, Cornelia de Lange, autosomal dominant mental retardation-5 and X-linked dominant early infantile epileptic encephalopathy-2.

Table 3. NIPT-SGA positive test rates for various groups of referrals.

Group*	No. Referrals	Positive results	% (95% CI)
Positive family history	132	20	15.2 (10.0-22.3)
All cases with abnormal ultrasound	514	99	19.3 (16.1-22.9)
Long bone abnormality	178	60	33.7 (27.2-40.9)
Cranial/facial	21	6	28.6 (13.6-50.2)
Lymphatic	150	20	13.3 (8.7-19.8)
Cardiac	31	4	12.9 (4.5-29.5)
Other ultrasound findings or unspecified	134	9	6.7 (3.4-12.4)
Advanced paternal age	912	2	0.2 (0.0-0.9)
Advanced maternal age/Other/unspecified	650	4	0.6 (0.2-1.6)
TOTAL	2,208	125	5.7 (4.8-6.7)

*See methods for details on how cases with cases with multiple findings were classified.

Table 4. Gestational ages for cases receiving testing because of ultrasound abnormality

	NIPT-SGD positive (%)	NIPT-SGD Negative (%)†	All cases with ultrasound abnormality (%)
1st trimester referrals	7 (7.1)	87 (21.8)	94 (18.8)
2nd trimester referrals	62 (62.6)	223(55.6)	285 (57.1)
3rd trimester referrals	30 (30.3)	90 (22.5)	120 (24.0)
TOTAL	99 (100)	400 (100)	499 (100%)
Mean gestational age (weeks)	24.3	21.5	22.1

†15 negative cases referred due to abnormal ultrasound had incorrect gestational ages (GA) and were excluded.

GA trimesters were based on ACOG guidelines i.e., 1st trimester 0-13.9 weeks, 2nd trimester 14-27.9 weeks, and 3rd trimester 28-40.9 weeks.