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Maternal carrier screening with single-gene NIPS provides accurate fetal risk assessments for recessive conditions

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ABSTRACT

Purpose: The purpose of this study was to evaluate the clinical performance of carrier screening for cystic fibrosis, hemoglobinopathies, and spinal muscular atrophy with reflex single-gene noninvasive prenatal screening (sgNIPS), which does not require paternal carrier screening.

Methods: An unselected sample of 9151 pregnant individuals from the general US pregnant population was screened for carrier status, of which 1669 (18.2%) were identified as heterozygous for one or more pathogenic variants and reflexed to sgNIPS. sgNIPS results were compared with newborn outcomes obtained from parent survey responses or provider reports for a cohort of 201 pregnancies.

Results: Overall, 98.7% of pregnant individuals received an informative result (no-call rate = 1.3%), either a negative carrier report or, if identified as heterozygous for a pathogenic variant, a reflex sgNIPS report. In the outcomes cohort, the negative predictive value of sgNIPS was 99.4% (95% CI = 96.0%-99.9%) and average positive predictive value (PPV) of sgNIPS was 48.3% (95% CI = 36.1%-60.1%). Importantly, personalized PPVs accurately reflected the percentage of affected pregnancies in each PPV range, and all pregnancies with a sgNIPS fetal risk of >9 in 10 (90% PPV) were affected.

Conclusion: Although traditional carrier screening is most effective when used to assess reproductive risk before pregnancy, more than 95% of the time it is pursued during a pregnancy and is complicated by incomplete uptake of paternal carrier screening (<50%) and misattributed paternity (~10%). Even in an idealized setting, when both partners have carrier screening, the maximum risk for having an affected pregnancy is 1 in 4 (equivalent of a 25% PPV). Carrier screening with sgNIPS during pregnancy is an alternative that does not require a paternal sample and provides accurate fetal risk in a timely manner that can be used for prenatal counseling and pregnancy management.

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Introduction

Offering prenatal carrier screening for common single-gene recessive disorders, including cystic fibrosis (CF), alpha- and beta-hemoglobinopathies, and spinal muscular atrophy (SMA), to all pregnant individuals or individuals pursuing a pregnancy is recommended by the American College of Obstetricians and Gynecologists (ACOG).¹ These disorders present in the newborn period and require lifelong medical management. Identification of high-risk pregnancies through prenatal carrier screening is critical to provide appropriate counseling for prenatal diagnostic testing, pregnancy care, and neonatal management.

However, more than half of fetuses at risk for one of these disorders because of parental carrier status is not identified by traditional carrier screening because the workflow relies on knowing both maternal and paternal carrier status. Although traditional carrier screening is recommended to be completed before pregnancy to allow for the most complete reproductive options,¹ this occurs in fewer than 5% of patients.² When carrier screening is completed during pregnancy, sequential screening (maternal carrier screening followed by paternal carrier screening) is the most common mode, resulting in an extended timeline, which may further reduce reproductive options.³ Furthermore, traditional carrier screening is limited by low uptake of paternal carrier screening, which is completed in fewer than half (41.5%) of the times it is indicated,^{2,4} and mis-attributed paternity, which occurs in approximately 10% (0.8%-30%) of pregnancies.^{5,6} These limitations of the traditional carrier screening workflow can result in low end-to-end sensitivity for identifying a high-risk pregnancy and provide limited information regarding fetal risk for these inherited disorders. Even when both maternal and paternal carrier status is known, the calculated risk is the couple's reproductive risk and is not tailored to the actual pregnancy.

Carrier screening with reflex single-gene noninvasive prenatal screening (sgNIPS) provides an alternative to address the inefficiencies of traditional carrier screening when applied to a pregnant person, by assessing the maternal carrier status and fetal risk from a single maternal blood draw, without the need for paternal carrier screening.⁷ The screen first assesses maternal carrier status for genes associated with the most common single-gene recessive disorders, including CF (*CFTR*), sickle cell disease and beta-thalassemia (*HBB*), alpha-thalassemia (*HBA1* and *HBA2*), and SMA (*SMN1*), using next-generation sequencing (NGS) of genomic DNA extracted from the buffy coat of a maternal peripheral blood sample (Supplemental Table 1). If the pregnant person is identified as heterozygous for a pathogenic variant in one or more of these genes, the sample is reflexed to sgNIPS, in which NGS is performed on the cell-free DNA (cfDNA) extracted from the original blood sample to determine the fetal risk. In approximately 14 to 21 days, the ordering provider receives the maternal carrier result and fetal risk together in 1 report.

In this study, we evaluated the clinical performance of carrier screening with reflex sgNIPS for identifying fetuses at risk for CF, alpha- and beta-hemoglobinopathies, and SMA in an unselected sample of pregnant individuals in the United States by comparing sgNIPS results with prenatal diagnostic testing, when available, and newborn outcomes. We found that carrier screening with sgNIPS is a more effective alternative to traditional carrier screening when a person is pregnant, with the clear benefits of identifying high-risk fetuses and providing tailored sgNIPS fetal risks in a timely manner without the need for a paternal sample.

Materials and Methods

Sample

We included pregnancies that had UNITY carrier screening with reflex sgNIPS ordered from BillionToOne, Inc, a Clinical Laboratory Improvement Amendments-certified clinical laboratory in Menlo Park, California, between August 5, 2019 and May 4, 2021. Pregnancies that were included had an estimated due date before June 1, 2021 or known fetal diagnosis, had sufficient sample, were a singleton gestation, and had a gestational age of ≥ 10 weeks. Pregnancies conceived using a donor egg or carried by a gestational carrier and pregnancies from international clinics and clinics involved in additional, ongoing clinical studies with BillionToOne were excluded. Sample processing and maternal carrier screening were completed according to clinical protocol (Supplemental Methods 1 and 2; Supplemental Table 1).

sgNIPS

The UNITY sgNIPS laboratory-developed test generates a likelihood ratio that compares the likelihood of the fetus inheriting a pathogenic genotype vs a nonpathogenic genotype.⁷ The likelihood ratio is calculated from the following cfDNA sequencing data: (1) fetal fraction, which is calculated by amplifying polymorphic alleles, identifying paternal alleles, and determining the allele fraction of the paternal alleles, (2) molecular counts of cfDNA using Quantitative Counting Templates,⁷ (3) variants that are not present in the maternal genotype (ie, paternally inherited variants), which are detected through amplification and sequencing of select regions of the *CFTR*, *HBB*, and *HBA* genes, and (4) maternal variant fraction, which is calculated by performing dosage analysis on *HBB* exon 1, *CFTR* F508del, and *SMN1* single copy variants. (Supplemental Method 3).

The likelihood ratio and the a priori risk were used to calculate a personalized, numerical residual disease risk for the fetus, referred to as the sgNIPS fetal risk. For most samples in which paternal carrier status is unknown, general US population prevalence sets the a priori risk. For each assay, a personalized risk reduction multiplier was applied to the a priori risk to set a minimum risk. When both parents

are known to be heterozygous for a pathogenic variant for a particular disorder (high-risk couples [HRCs]), then the a priori risk was set at 1 in 4 and the risk reduction multiplier was set at a maximum of 100-fold, capping the sgNIPS fetal risk for HRCs at 1 in 400 at the lowest end. A no call was reported when information from sgNIPS was uninformative and no repeat sample was received, or when sgNIPS was uninformative on 2 samples.

Newborn outcomes data collection via quality assurance program

A quality assurance (QA) program was established in August, 2020 to evaluate concordance between the sgNIPS risk results and fetal/neonatal clinical outcomes. All individuals with reflex sgNIPS were included in the QA program. Patients were contacted directly through a combination of telephone calls and text messaging a minimum of 45 days after reported estimated delivery date for answering questions specific to newborn outcomes for the condition(s) for which sgNIPS was performed. Up to 3 contact attempts were made and surveys were sent via text. Newborn information, including date of birth, newborn screen results (positive or negative for condition of interest), additional testing related to the condition of interest (ie, prenatal diagnostic testing, postnatal molecular testing, and partner carrier screening), newborn or pediatric symptoms of concern, and reports of referrals to pediatric specialists, for each case was collected through verbal report or survey. Although state newborn screening (NBS) programs include screening for CF and hemoglobinopathies, 17 states did not include SMA in their NBS programs during our recruitment period (before June 1, 2021).⁸ Therefore, concordance for SMA relied on either newborn screen results or presence/absence of SMA characteristics, report of additional pediatric testing, and specialty referrals. In some cases, providers contacted the laboratory to provide clinical outcome information, particularly in the cases in which prenatal diagnostic testing was performed. Publication of the QA program summary data was approved by an independent institutional review board (WCG ID 13472102).

Analysis

Summary data were compiled from the QA program collected from pregnancies with an estimated due date before June 1, 2021, as well as any unsolicited clinical outcomes reported by providers. Together, these cases comprised the outcomes cohort ($n = 201$). Pregnancies without outcome information or no-call sgNIPS risk results ($n = 10$) were excluded from the outcome analysis ($n = 191$). In actual practice, each sgNIPS report provides a personalized fetal risk estimate computed from the log likelihood ratio of the pregnancy inheriting 2 pathogenic variants. In the outcome analysis, the risk estimates were stratified into fetal risk of ≥ 1 in 100 (high risk) and < 1 in 100 (low risk) (Supplemental

Table 2). The sgNIPS risk was considered accurate if the sgNIPS was low risk and fetus/newborn was reported as unaffected with the condition of interest and if the sgNIPS result was high risk and the fetus/newborn was reported as affected with the condition of interest. Clinical analytics for sgNIPS, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and correlation of PPV with sgNIPS fetal risk, were computed. The 95% CIs were the Clopper-Pearson values (sensitivity and specificity) or the standard logit values (predictive values).⁹

The end-to-end sensitivity for carrier screening with sgNIPS to correctly identify an affected pregnancy as high risk (ie, for the complete cohort of 9151 pregnancies reflecting the combined performance of carrier screening and sgNIPS together) was calculated using the sensitivity of carrier screening, the sensitivity of sgNIPS from the outcomes cohort, and the overall no-call rate (Supplemental Methods 4 and 5). We also calculated the sensitivity for traditional carrier screening to identify a HRC and therefore a high-risk pregnancy, assuming a 41.5% paternal screening rate^{2,4} and a 10%^{5,6} misattributed paternity rate. These values ranged in the literature, and an intermediate value was selected (Supplemental Method 6). The end-to-end specificity of carrier screening with sgNIPS was calculated from the end-to-end sensitivity, the estimated number of affected fetuses given the maternal carrier frequency, population carrier frequency, and number known of HRCs (Supplemental Method 6). Analyses were completed in Microsoft Excel (Version 16.4, Microsoft Corporation) and online calculator MedCalc https://www.medcalc.org/calc/diagnostic_test.php (Version 19.4, MedCalc Software).

Results

Characteristics of the complete cohort, reflex sgNIPS cohort, and outcomes cohort

Our complete cohort consisted of 9151 pregnant individuals from 31 US states from more than 240 providers. Of these, 1669 individuals (18.2%) were heterozygous for a pathogenic variant for at least 1 condition and reflexed to sgNIPS (Figure 1). A total of 1833 sgNIPS assays were performed; 156 individuals were heterozygous for a pathogenic variant in 2 conditions, and 4 individuals were heterozygous for a pathogenic variant in 3 conditions. In the reflex sgNIPS cohort, 4.47% were heterozygous for *CFTR* pathogenic variants, 4.64% for *HBB* variants, 8.65% for *HBA1/HBA2* variants, and 2.26% for *SMN1* exon 7 deletion (Table 1). These observed carrier frequencies are 0.4 to 1.6% higher than the general US population,^{10,11} suggesting that the unselected population was enriched for individuals heterozygous for a pathogenic variant in one or more of the 4 conditions.

Of the 9151 individuals in the complete cohort, 98.7% received a negative carrier report or, if they were found to be heterozygous for one or more pathogenic variants, received a

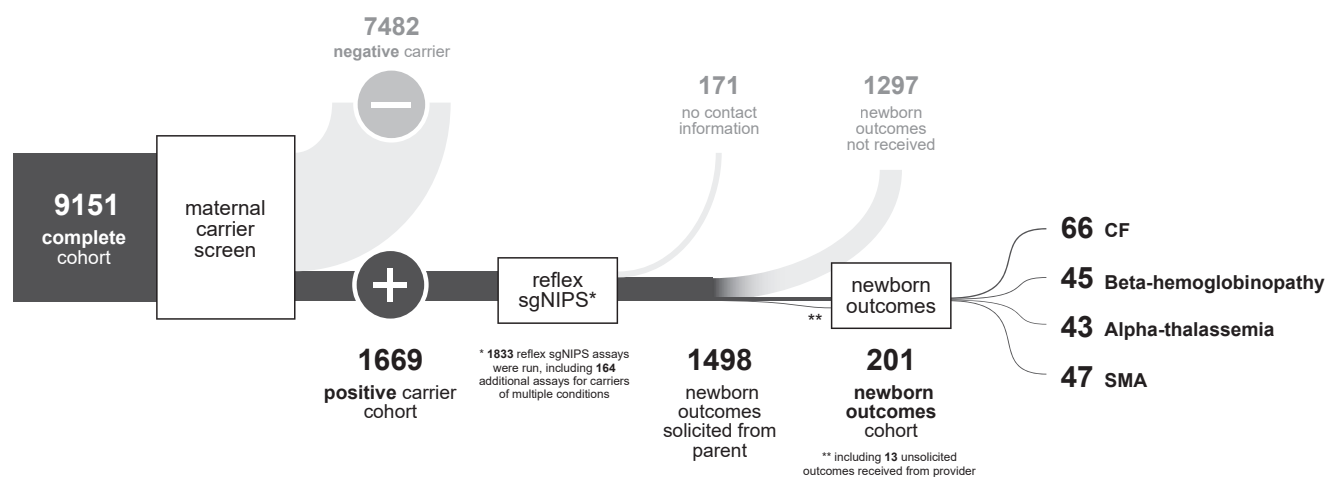


Figure 1 Study cohort assembly. Numbers of study participants in the complete cohort, reflex sgNIPS cohort, and newborn outcomes cohort. CF, cystic fibrosis; sgNIPS, single-gene non-invasive prenatal screening; SMA, spinal muscular atrophy.

sgNIPS fetal risk report (no-call rate = 1.3%). Reflex sgNIPS results included a personalized, numerical sgNIPS fetal risk and a corresponding risk category (high risk, increased risk, decreased risk, or low risk) (Supplemental Table 2). Of the pregnancies with a general population a priori risk, 96.5% had an sgNIPS risk of <1 in 100 and 1.9% had a sgNIPS risk of >1 in 4. The others either had an sgNIPS increased risk (1.3%) that fell between a priori and 1 in 4 or a decreased sgNIPS risk (0.3%) that fell between 1 in 100 and the general population a priori risk. Of the 57 pregnancies belonging to HRCs in which the couple's a priori reproductive risk was 1 in 4, 60% received an sgNIPS risk of <1 in 100 and 28% received an sgNIPS risk of >1 in 4. Only 7 (12%) had an sgNIPS risk between 1 in 4 (a priori) and 1 in 100.

In the reflex sgNIPS cohort, clinical outcome data was requested as part of the QA program from 1488 individuals with contact information and 188 outcomes were obtained (12.6% response rate). An additional 13 unsolicited clinical outcomes were reported by the ordering provider summing a total of 201 outcomes: 66 CF outcomes, 45 beta-hemoglobinopathy outcomes, 43 alpha-thalassemia outcomes, and 47 SMA outcomes (Figure 1). Of the 201 pregnancies with outcome data, sgNIPS reported a fetal risk for 191 (95%) and a no result, meaning a fetal risk was not

returned for 10 (5.0%), slightly higher than that reported for the full sample.

The reflex sgNIPS cohort had a mean gestational age of 16 weeks 6 days \pm 6 weeks 1 day, a mean maternal age of 28.5 \pm 5.9 years, and a mean fetal cfDNA fraction of 6.8% \pm 4.3% (Supplemental Table 3). The mean gestational age, maternal age, and fetal fraction were not significantly different between the full reflex sgNIPS cohort and the outcomes cohort (Supplemental Figure 1). The outcomes cohort had more high/increased risk reports than the full reflex sgNIPS cohort (13.4% vs 4.3%). High-risk case enrichment and the presence of HRCs in the cohort can impact the clinical analytics and result in artificially lower specificity and NPV of sgNIPS.

Clinical analytics for carrier screening with reflex sgNIPS for all conditions

In the outcomes cohort, sgNIPS identified 14 out of 15 affected fetuses as high risk for one of the conditions on the screening panel, resulting in a sensitivity of 93.3% (95% CI = 68.1%-99.8%) (Table 2, Supplemental Table 4). Of the 162 low-risk results, 161 were unaffected, resulting in an

Table 1 Description of study cohort ($N = 9151$)

Total Cohort	Reflex sgNIPS Cohort	Total sgNIPS Assays	Per Condition			
			CF	HBB	HBA	SMA
Total (N)	1669	1833	409	425	792	207
Carrier rate (%) ^a	18.24		4.47	4.64	8.65	2.26
Reported risk (n)						
High		48	11	24	0	13
Increased		30	7	2	9	12
Decreased		79	12	52	1	14
Low		1554	358	316	741	139
No call		122	21	31	41	29

CF, cystic fibrosis; HBA, alpha hemoglobinopathy; HBB, beta hemoglobinopathy; SMA, spinal muscular atrophy; sgNIPS, single-gene noninvasive prenatal screening.

^aFor comparison, the general US population carrier rates are 3.85% (CF), 3.03% (HBB), 7.69% (HBA), and 1.85% (SMA).^{10,11}

Table 2 sgNIPS fetal risk compared with newborn outcomes

Newborn Outcome	sgNIPS Fetal Risk		Row Total
	High	Low	
Affected	14	1 ^a	15
Unaffected	15	161	176
Column total	29	162	191 ^b
Clinical Analytics	Value	95% CI	
Sensitivity	93.3%	68.1%-99.8%	
PPV	48.3%	36.1%-60.1%	
NPV	99.4%	96.0%-99.9%	

NPV, negative predictive value; PPV, positive predictive value; sgNIPS, single-gene non-invasive prenatal screening.

^aThe single discordant call was due to a rare CF variant (G542X) homozygous case, which sgNIPS is not designed to detect. sgNIPS, as designed, detects the common homozygote cases across *HBB*, *CFTR*, and *SMN1* genes, as well as any compound heterozygote cases (including with G542X) but not homozygote cases for rare CF variants. Together, all rare variant homozygous cases are expected to account for 1.5% to 3.1% of North American CF cases.^{12,13}

^bTotal does not include 10 cases with a no-call returned (3 CF, 1 HBB, and 6 SMA); all no calls were unaffected.

NPV of 99.4% (95% CI = 96.0%-99.9%) (Table 2). The single discordant low-risk call was a rare case that the sgNIPS is not designed to detect. This was reflected in the sgNIPS fetal risk reported as 1 in 2000. See the following “sgNIPS fetal risk for CF” section for further description. Of the 148 non-HRCs with low-risk results, 22 (15%) reported pursuing partner carrier screening. Of the 29 high-risk results, 14 were affected, resulting in a PPV of 48.3% (95% CI = 36.1%-60.1%) (Table 2).

The personalized sgNIPS fetal risk correlated with the percentage of fetuses affected, indicating an accurate risk-level assessment. Across all conditions in the outcomes cohort, 4 out of 4 (100%) pregnancies with >9 in 10 risk were affected, 8 out of 17 (47%) with risks between 1 in 2 and 2 in 3 risk were affected, 2 out of 8 (25%) with risks between 1 in 10 and 1 in 100 were affected, and 1 out of 162 (0.6%) with risks <1 in 100 were affected (Figure 2A).

sgNIPS fetal risk for CF

In the outcomes cohort, more than half of the individuals who were heterozygous for a pathogenic variant in the *CFTR* gene (59%) had the F508del pathogenic variant, consistent with the published prevalence of this variant in the US¹² (Appendix 1). Out of 66 individuals who were heterozygous for a pathogenic variant, 9 had high-risk results (Figure 2B, Supplemental Table 4). Three had a sgNIPS fetal risk of >9 in 10, and all 3 were affected. Two of these were homozygous for the F508del pathogenic variant, and 1 was compound heterozygous with maternal F580del pathogenic variant. Out of the remaining 6 high-risk cases, 1 had a 6 in 10 risk for homozygous F508del pathogenic variant based on dosage analysis, and the newborn was affected. In the other 5 cases, a pathogenic paternal variant was detected, resulting in a risk of 1 in 2. Two of these 5 cases were affected, consistent with the sgNIPS fetal risk of 1 in 2.

There were 47 CF cases with low-risk results, with sgNIPS fetal risks ranging from 1 in 200 to 1 in 2800 (Figure 2B; Supplemental Table 4). Out of the low-risk cases,

46 were unaffected and 1 was affected (Figure 2B, case 43). In this case, the mother was heterozygous for the pathogenic variant G542X, which has a frequency of 2.56% among individuals heterozygous for *CFTR* variants.¹² Because no paternal pathogenic variant was detected, the sgNIPS fetal risk was reduced to 1 in 2000 (Supplemental Method 7). NBS revealed that the neonate was homozygous for the G542X pathogenic variant and therefore affected with CF. The CF sgNIPS NGS-based dosage analysis is designed to detect F508del homozygous cases and compound heterozygous cases (including G542X) but not homozygous cases for rare *CFTR* variants. Together, all rare variant homozygous cases are expected to account for 1.5% to 3.1% of US CF cases, with the higher end of the range impacted by assortative mating.^{12,13}

sgNIPS fetal risk for beta-hemoglobinopathies

Individuals who were heterozygous for hemoglobin S (sickle cell) hemoglobin C, hemoglobin E and beta-thalassemia pathogenic variants were all represented in the outcomes cohort (Appendix 1). Of 40 individuals who were heterozygous for beta-hemoglobinopathies, 6 had pregnancies with high risk sgNIPS result. One had an sgNIPS fetal risk of >9 in 10 and was affected with sickle cell disease (genotype homozygous hemoglobin S pathogenic variant) (Figure 2C). Four had a 1 in 2 sgNIPS fetal risk because a pathogenic paternal allele was detected but dosage for the maternal allele was either not available or not informative. Two were affected and 2 were unaffected, consistent with the sgNIPS fetal risk of 1 in 2 (Figure 2C). The last high-risk case fell near the high-risk cutoff with an sgNIPS fetal risk of 1 in 92, and the newborn was unaffected.

All 34 low-risk cases were unaffected (Figure 2C; Supplemental Table 4). Because the a priori risk for beta-hemoglobinopathies (1 in 32) was the highest among the a priori risks for all disorders on the reflex sgNIPS panel, sgNIPS fetal risks spanned a larger range, with several low-risk cases falling in the 1 in 200 to 1 in 2000 range. In all

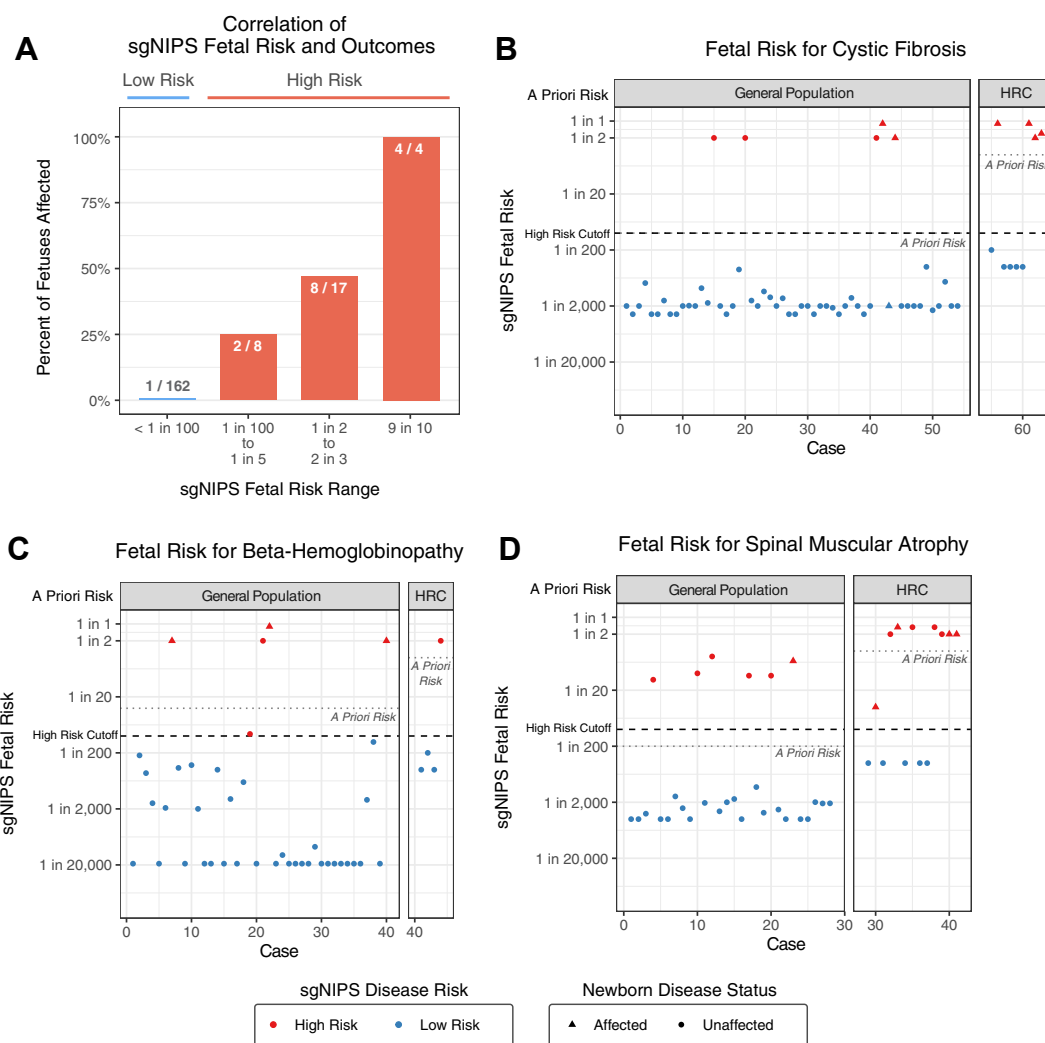


Figure 2 sgNIPS fetal risk for ACOG-recommended single-gene recessive disorders. A. sgNIPS fetal risk was binned into 4 categories (<1 in 100, 1 in 100 to 1 in 5, 1 in 2 to 2 in 3, and >9 in 10) and correlated with the percentage of affected fetuses in the outcomes cohort. Numbers used to calculate proportion affected (number affected by total number in risk category) are indicated in each column. B-D. Numerical sgNIPS fetal risk and corresponding risk category (high risk, 1 in 100 or low risk, <1 in 100) for pregnancies at risk for cystic fibrosis (CF) (B), beta-hemoglobinopathy (HBB) (C), and spinal muscular atrophy (SMA) (D). Newborn disease status (affected or unaffected) was collected from parental or provider report of diagnostic testing or newborn screening (NBS). For most pregnancies, the a priori risk was determined by the general population carrier frequency: 1 in 100 for CF (B); 1 in 32 for HBB (C); and 1 in 200 for SMA (D). The minimum reported risk was 1 in 2800 for CF (B), 1 in 19,000 for HBB (C), and 1 in 4000 for SMA (D). For pregnancies belonging to HRCs, the a priori risk was 1 in 4 and the minimum risk was 1 in 400. Cases reported clinically as increased-risk and decreased-risk were classified for this analysis as high risk or low risk based on the sgNIPS fetal risk. For CF, 7 cases clinically reported as decreased-risk were classified as low-risk for this analysis (B). For HBB, 11 cases were clinically reported as decreased-risk (C). Ten were classified as low-risk and 1 was classified as high risk for this analysis. For SMA, 6 cases clinically reported as increased-risk were classified as high risk (D). Six cases were clinically reported as decreased-risk, including 5 that were classified as low-risk and 1 that was classified as high risk for this analysis. There were no high risk or affected cases for alpha-thalassemia sgNIPS (data shown in Supplemental Figure 2). ACOG, American College of Obstetricians and Gynecologists; sgNIPS, HRC, high-risk couple; single-gene non-invasive prenatal screening.

cases, sgNIPS provided sufficient data to estimate the fetal risk either higher or lower than the a priori risk.

sgNIPS fetal risk for alpha-thalassemia

The 43 alpha-thalassemia cases in the outcomes cohort were all classified as low risk and had sgNIPS fetal risks of 1 in 7100 or lower (Supplemental Figure 2; Supplemental

Table 4; Appendix 1). All were unaffected, leading to 100% concordance. All 4 types of alpha-thalassemia pathogenic variant combinations were represented in our cohort, including 35 individuals who had 3 functional copies of *HBA1/HBA2*, 6 individuals who had 2 functional copies of the *HBA1/HBA2* gene in trans, 1 individual who had 2 functional copies of *HBA1/HBA2* in cis, and 1 individual who had the Hemoglobin Constant Spring pathogenic variant

(inactivating pathogenic variant on 1 copy of *HBA2*). Individuals with pathogenic variants in cis had a higher a priori risk (1 in 186) than other individuals with pathogenic variants in trans or 3 functional copies of *HBA1/HBA2* (1 in 372) because the double deletion is inheritable (Supplemental Figure 2).

Although alpha-thalassemia is the least prevalent condition returning the fewest high-risk reflex sgNIPS results, it was the most common sgNIPS performed because it has the highest carrier frequency. In our complete cohort, 792 samples (8.65%) were reflexed to sgNIPS for alpha-thalassemia compared with 425 samples (4.64%) reflexed to sgNIPS for the next most common condition, beta-hemoglobinopathies (Table 1).

sgNIPS fetal risk for SMA

Of the 41 pregnant individuals who were heterozygous for an *SMN1* gene deletion, all 27 fetuses classified as low risk were unaffected (Figure 2D; Supplemental Table 4; Appendix 1). In total, 14 fetuses were classified as high risk and 5 were affected. One of the 3 cases with a 2 in 3 sgNIPS fetal risk was affected, 2 of the 4 cases with a 1 in 2 sgNIPS fetal risk were affected, and 2 of the 7 cases with 1 in 5 to 1 in 50 sgNIPS fetal risk were affected (Figure 2D). As seen with the other assays, the percentage of affected fetuses correlated with the sgNIPS fetal risk range.

Calculated end-to-end clinical analytics for fetal risk assessment

Because sgNIPS is always coupled with the carrier screening workflow, we calculated the estimated end-to-end clinical analytics to reflect the performance of the 2 step analysis to accurately assess fetal risk. For comparison, we performed a similar analysis for traditional carrier screening applying different scenarios. Both carrier screening with reflex sgNIPS and traditional carrier screening were affected by the sensitivity of carrier screening itself. For our simulation, we used the sensitivity of best-in-class NGS carrier screening, which differs for each condition (for CF, >99%; beta-hemoglobinopathy, >99%; alpha-thalassemia, >95%; SMA, >91.3%) and has a weighted average of 96.4% for all conditions combined (Supplemental Tables 1 and 5; Supplemental Methods 4 and 6). In a hypothetical scenario with 0% misattributed paternity and 100% paternal screening uptake, traditional carrier screening has an end-to-end sensitivity of 93% to identify an HRC and therefore increased risk for an affected pregnancy, based solely on the multiplication of sensitivity of carrier screening for 2 parents (Figure 3).⁵ In a best-case scenario with 100% paternal screening uptake but misattributed paternity in 10% of cases^{5,6} (causing erroneous fetal risk assessments), the end-to-end sensitivity drops to 84%. In a US-average, real-world scenario in which paternity may be misattributed in 10% of cases and paternal carrier screening may not be performed in

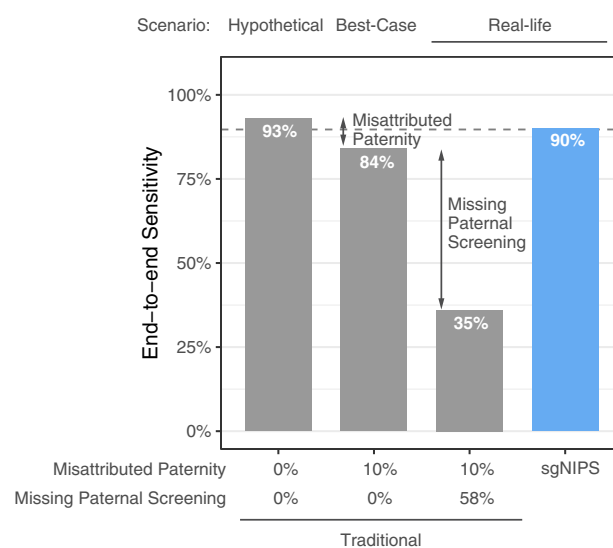


Figure 3 Calculated clinical performance of prenatal carrier screening. Estimated end-to-end sensitivity for traditional carrier screening (in hypothetical, idealized, and real-world scenarios) and for carrier screening with reflex single-gene noninvasive prenatal screening.

58%^{2,4} of cases, the end-to-end sensitivity further drops to 35% (Figure 3). Because carrier screening with reflex sgNIPS does not rely on paternal screening, its sensitivity is not affected by misattributed paternity or paternal screening uptake. In a real-world scenario that accounts for the sensitivity of carrier screening and the sensitivity of sgNIPS, the end-to-end sensitivity of carrier screening with reflex sgNIPS was 90.0% (95% CI = 71.8%-98.9%) (Figure 3). This end-to-end sensitivity is higher than that of traditional screening in the real-world scenario, higher than the best-case scenario, and similar to the hypothetical scenario.

We calculated the estimated end-to-end specificity for carrier screening with reflex sgNIPS for the complete cohort using our outcomes cohort data (Supplemental Method 6). The estimated end-to-end specificity for carrier screening with reflex sgNIPS, including the negative carrier results, is 99.8% (95% CI = 99.6%-99.9%) (Supplemental Table 5). Because specificity is cohort-specific, this value is more reflective of overall test performance than specificity calculated from the high-risk enriched outcomes cohort.

Discussion

This clinical study demonstrated the validity of maternal carrier screening with reflex sgNIPS for the ACOG-recommended single-gene recessive conditions in an unselected clinical sample of 9151 pregnant individuals. On assessing the accuracy of sgNIPS to identify affected pregnancies as high risk in the 201 cases with fetal or neonatal outcomes, we found that carrier screening with sgNIPS provided a timely, accurate, personalized fetal risk estimate without the need for a paternal sample and may

serve as an alternative that is not subject to the inefficacies of traditional carrier screening.

In both traditional carrier screening and carrier screening with reflex sgNIPS, a positive maternal carrier screen increases the a priori risk of having an affected fetus. In our study, reflex sgNIPS yielded an average PPV of 48.3% (95% CI = 36.1%-60.1%) without the need for paternal carrier screening. This is higher than the best-case 25% PPV that traditional carrier screening can achieve during a pregnancy with complete paternal follow-up and no mis-attributed paternity. It compares similarly to even the most specific cfDNA-based aneuploidy screens for average-risk pregnancies, which has a PPV of 38% to 80% for trisomy 21, 11% to 41% for trisomy 18, and 5% to 13% for trisomy 13 at the maternal age of 20 years.¹⁴ Importantly, the personalized sgNIPS fetal risk strongly correlated with the percentage of affected pregnancies; all cases with a risk of >9 in 10 were affected, whereas only one of the 162 cases with a risk of <1 in 100. These data demonstrate how the numerical value of the fetal risk reflects the certainty of the sgNIPS assay's estimation of the fetal outcome. The informative personalized sgNIPS fetal risk can be used to guide clinical decision-making about diagnostic testing and follow-up as is evident by the report of partner carrier screening in only 15% of cases with sgNIPS risk results <1 in 100.

The sensitivity of sgNIPS was 93.3% (95% CI = 68.1%-99.8%), and the estimated end-to-end sensitivity (including carrier screening) was 90% (95% CI = 71.8%-98.9%). This end-to-end sensitivity of carrier screening with sgNIPS is higher than the estimated real-world end-to-end sensitivity of traditional carrier screening completed during pregnancy, which is only 35%. Given the published data on US mis-attributed paternity and paternal screening uptake rates, traditional carrier screening may be ineffective in many cases and implementation of carrier screening with reflex sgNIPS workflow, which does not require a paternal sample, may identify higher number of affected fetuses as at high risk.

The implementation of carrier screening with reflex sgNIPS workflow during pregnancy might be important to improve health care equity. Individuals of lower socioeconomic status or who identify as ethnic or racial minorities are disproportionately affected by factors that affect the effectiveness of traditional carrier screening.¹⁵⁻¹⁸ These include cases when the pregnancy is unplanned, late presentation to prenatal care, no relationship with the partner, or the partner has other barriers that make it difficult to pursue carrier screening. For example, in the US, approximately 4 in 10 pregnant individuals are covered by Medicaid or managed Medicaid.¹⁵ This coverage is available to all qualified, uninsured individuals throughout a pregnancy, but this same benefit does not extend to the father of the pregnancy. This limits their access to medical care and creates logistical and financial barriers to carrier screening.

In our complete cohort, we observed carrier frequencies slightly higher than those in the general US obstetric population, likely because of the enrichment of individuals who were known to be heterozygous for a pathogenic variant in one of these conditions and HRCs. This suggests ordering providers used the assay to risk stratify known high-risk pregnancies and not just as a first-line carrier screen. The sgNIPS assay returned a fetal risk of either >1 in 4 or <1 in 100 for 88% of the HRCs. Our performance data demonstrates that sgNIPS is robust for this real-world clinical use.

We previously reported on the high sensitivity and specificity of the sgNIPS HBB assay for sickle cell disease.¹⁹ This study demonstrated that the HBB assay continued to have high performance when all common HBB heterozygous pathogenic variants were included. The HBB gene is relatively small and most pathogenic variants are single-nucleotide variations, resulting in high confidence sgNIPS calls that translate into well-separated risk estimates from the a priori risk.

sgNIPS for alpha-thalassemia may be particularly impactful for the health care system, because severe disease is rare (<0.07%) but carrier frequencies are high. The carrier frequency in the United States is 1 in 16, and most have 3 functional copies of the *HBA1/HBA2* gene.^{10,11,20} A pregnancy is only at risk for severe disease if at least 1 partner has 2 *HBA1/HBA2* gene deletions or single-nucleotide variations, which occurs at a frequency of 1 in 570 in the general US population but varies widely with ethnicity, from approximately 1 in 5000 for African Americans to >1 in 100 for Asians.¹¹ Given these frequencies, partner screening usually reveals the pregnancy as low risk. The low uptake of paternal carrier screening results in many low-risk pregnancies remaining at risk because paternal status is unknown. Alternatively, sgNIPS provides a fetal risk for all pregnancies without paternal screening, reducing cost and burden for the health care system¹³ and resulting in a more personalized risk assessment for the pregnant individual.

A rare limitation of sgNIPS assay sensitivity was demonstrated by the case in which low-risk results were issued and the neonate was identified as homozygous G452X, affected with CF. In the CF sgNIPS assay, NGS is performed to cover all critical *CFTR* exons, but dosage analysis is not performed for all exons. This means that the CF sgNIPS can detect F508del homozygous cases and any compound heterozygous cases, but not homozygous cases for non-F508del (which account for 1.5%-3.1% of US CF cases).^{12,13} Importantly, the estimated personalized sgNIPS risk reflects these assay limitations and the result report and test literature include a discussion of it. In this case, sgNIPS risk for individuals who are heterozygous for non-F508del pathogenic variants is only as low as 1 in 2000, which accounts for the probability that the fetus is homozygous for a rare non-F508del pathogenic variant. Pregnant individuals should have appropriate pre- and post-test counseling regarding the benefits and limitations of the assay, including the potential for reduced performance in cases of parental

consanguinity or shared ancestries, particularly those with known genetic homogeneity.

The sgNIPS SMA assay is more challenging than other sgNIPS assays because it measures copy number, resulting in a lower signal-to-noise ratio, and sequencing is complicated by high homology between the *SMN1* and *SMN2* genes. Despite these challenges, it had excellent sensitivity in the outcomes cohort and identified all 5 SMA affected fetuses to be at high risk. The no-call rate was the highest of all the assays because a high threshold for molecular counts was set to improve signal-to-noise. The SMA sgNIPS assay used in this study outperformed traditional carrier screening, and improvements to the assay that were implemented after this study are expected to lower its no-call rate.

Limitations

In the complete cohort, 98.7% of pregnant individuals received a negative carrier screening result or an sgNIPS result, clarifying risk and enabling streamlined management. For the 1.3% who received a no call result, all were heterozygous for a pathogenic variant and received a no call on the sgNIPS assay. A no call is most often because of an inadequate number of fetal molecules in the cfDNA related to low genomic equivalents and/or fetal fraction. These values are impacted by fetal or maternal factors (particularly maternal weight,¹⁴ which was unavailable in this study) and sample compromise during transport. The sgNIPS no-call rate is likely inflated because the second maternal blood sample requested was not received for all initial no calls.

In our study, ethnicity data was unavailable. However, the sgNIPS assay is not personalized based on the ethnicity. Therefore, lack of ethnicity data does not affect the overall results, but collection could be helpful in the future for interpreting results and understanding assay performance across different ethnicities.

The clinical analytics calculated from our study may have been affected by a modest number of outcomes collected, which is reflected by the enrichment of high-risk sgNIPS cases in the outcomes cohort compared with the sgNIPS cohort. For example, the specificity calculated from the outcomes cohort is uninformative because of this enrichment; therefore, we calculated the end-to-end specificity to provide a more meaningful estimate. Of note, high specificity for the *HBB* sgNIPS was measured in a previous study in which outcomes were collected in an unenriched cohort.¹² Enrichment of the outcomes cohort for cases can inflate the PPV and deflate the NPV. Furthermore, it is also reasonable to hypothesize that outcomes that are not consistent with the sgNIPS risk estimate are more frequently reported than those that are consistent, which would artificially decrease the sensitivity calculated in this study. However, it is not possible to predict how all of these potential, but unknown, biases together affected the clinical analytics.

Outcomes, particularly for unaffected cases, were determined through NBS results rather than molecular diagnosis.

Although molecular diagnosis is the gold standard outcome, NBS (which is designed with a high sensitivity at the expense of specificity) is likely a good estimate of unaffected outcomes. For all the affected cases for CF and SMA, molecular testing was completed either as part of NBS or clinical indication. Most cases of hemoglobinopathies were identified using high performance liquid chromatography analysis of hemoglobin variants via NBS.

Future research inspired by the high clinical performance of carrier screening with reflex sgNIPS in this study includes continued evaluation of this assay in larger cohorts with more complete collection of fetal outcomes. In addition, studies exploring the effect of carrier screening with sgNIPS on clinical practice including a one-to-one comparison with traditional carrier screening and the patient and provider experience can further inform clinical implementation.

Conclusion

Carrier screening with sgNIPS is a more effective alternative to traditional carrier screening for many, but not all, individuals who have carrier screening after conception. For a significant proportion of individuals, the recommendation of pre-pregnancy traditional carrier screening is not an option (45% of pregnancies are unplanned).^{16,21} The effectiveness of using traditional carrier screening results to guide pregnancy decisions for diagnostic procedures is diminished by unpartnered individuals (20% to 40%)^{17,18} and late presentation to prenatal care (23% present after the first trimester).¹⁸ These factors are more common among individuals who are younger, have lower education levels, or identify as minorities, as a result, traditional carrier screening may contribute to health care disparities.^{16-18,21}

Expanded carrier panels available through traditional carrier screening pathways can identify additional conditions and therefore more HRCs. This may be a preferable option for some individuals particularly if the full range of diagnostic testing and pregnancy management options are desired. However, when performed after conception, as >95% of carrier screening is currently performed, the value is significantly diminished, especially if a partner is not available to test and/or an individual presents to pregnancy care at a late gestational age. Nonpregnant individuals, individuals early in their pregnancy with available partners who are of ancestries with known founder pathogenic variants (such as Ashkenazi Jewish ethnicities), or pregnancies with known consanguinity or family history of a specific autosomal recessive condition that is not included in the current panel will continue to benefit from traditional carrier screening options and expanded panels. However, sgNIPS can be expanded to include additional autosomal recessive conditions in the future.

This study demonstrated the high clinical performance of maternal carrier screening with reflex sgNIPS to identify fetuses at high risk for single-gene recessive disorders on the ACOG panel. Carrier screening with reflex sgNIPS may

improve prenatal detection of affected pregnancies compared with traditional carrier screening and does not require paternal screening. It provides an sgNIPS fetal risk tailored to the pregnancy, facilitating appropriate counseling for diagnostic testing and follow-up.

Data Availability

All data are available in the manuscript and supplementary material.

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Ethics Declaration

Publication of the quality assurance program summary data was approved by the WCG (<https://www.wcgclinical.com/>) independent Institutional Review Board (WCG ID 13472102). Informed consent was not required for this retrospective analysis of data collected as part of a quality assurance program.

Conflict of Interest

J.H., J.W., S.R., and J.R.t.B. are employees of BillionToOne and hold stock or options to hold stock in the company. J.A.C. is financially compensated by BillionToOne. E.E.H. received an educational grant from BillionToOne. A.N.T. and N.L.V. declare no conflicts of interest.

Additional Information

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